Attorney Docket No.: CONLINCO-03681

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Asgeir Saebo, *et al.*		
Serial No.:	09/271,024	Group No.:	1617
Filed:	03/17/99	Examiner:	Wang, S.
Entitled:	**Conjugated Linoleic Acid Compositions**		

TRANSMITTAL OF APPEAL BRIEF (PATENT APPLICATION - 37 CFR § 192)

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.8(a)(1)(i)(A)

I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being deposited with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop Appeal Brief - Patents, Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia, 22313-1450.

Dated: November 25, 2003 By: Susan M. McClintock
Susan M. McClintock

Sir or Madam:

1. Transmitted herewith, in triplicate, is the APPEAL BRIEF in this application, with respect to the Notice of Appeal filed on **August 25, 2003**.

2. **STATUS OF APPLICANT**

This application is on behalf of:

other than a small entity.

3. **FEE FOR FILING APPEAL BRIEF**

Pursuant to 37 CFR § 1.17(g), the fee for filing the Appeal Brief is:

Fee for Filing Appeal Brief: $330.00

4. **EXTENSION OF TERM**

The proceedings herein are for a patent application, and the provisions of 37 CFR § 1.136 apply.

Applicant petitions for a one-month extension of time under 37 CFR § 1.136 (fees: 37 CFR §§ 1.17(a)-(d)).

Fee for Extension of Time: $110.00

5. TOTAL FEE(s) DUE

The total fee due is:

Appeal brief fee: $330.00

Extension fee (if any): $110.00

TOTAL FEE DUE: $440.00

6. FEE PAYMENT

Attached is a check for **$440.00**.

7. FEE DEFICIENCY

If any additional fee is required, please charge Deposit Account No. **08-1290**.

Dated: November 25, 2003

J. Mitchell Jones
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PATENT
Attorney Docket No. **CONLINCO-03681**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Asgeir Saebo *et al.*
Serial No.: 09/271,024 Group No.: 1617
Filed: 03/17/99 Examiner: Wang, S.
Entitled: **CONJUGATED LINOLEIC ACID COMPOSITIONS**

APPELLANTS' BRIEF
APPEAL NO.:

Mail Stop Appeal Brief - Patents
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P.O. Box 1450
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Dated: November 25, 2003 By: Susan M. McClintock
Susan McClintock

Sir:

This Brief is in furtherance of the Notice of Appeal filed March 20, 2002.

The fees required under § 1.17(h) and any required Petition for Extension of Time for filing this Brief and fees therefore are dealt with in the accompanying TRANSMITTAL OF APPEAL BRIEF.

This Brief is transmitted in triplicate. [37 C.F.R. § 1.192(a)].

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This Brief contains these items under the following headings and in the order set forth below [37 C.F.R. § 1.192(c)]:

I. REAL PARTY IN INTEREST

The real party in interest is Natural ASA, a Norwegian Corporation.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences known to Appellants, Appellants' legal representative, or the Assignee.

III. STATUS OF THE CLAIMS

Claims 1 - 12 were filed in the original application. During prosecution of the application, Claims 13 - 17 were added, and Claims 1 - 4 and 9 - 11 were cancelled. Claims 5 - 8 and 13 - 17 have been rejected by the Office in the Advisory Action dated June 18, 2003. Therefore, Claims 5 - 8 and 13 - 17 are pending in this appeal. No other claims are pending. Thus, Appellants appeal the Final Office Action of March 26, 2003 and the Advisory Action of June 18, 2003.

The Claims, as they now stand, are set forth in Appendix A.

IV. STATUS OF THE AMENDMENTS

Appellants' Amendment and Response to the Final Office Action filed on May 27, 2003 has been entered per the Advisory Action dated June 18, 2003 (Paper No. 28). No amendments to the claims were made in that Response. Thus, all amendments, including those made Applicant's Response dated December 13, 2002.

V. SUMMARY OF THE INVENTION

The present invention relates to the field of human and animal nutrition, and in particular to novel compositions of conjugated linoleic acid (CLA). The compositions are prepared by novel methods that control isomerization of linoleic acid. In particular, the present invention is directed to compositions that comprise acylglycerides that comprise CLA isomers at one or more of the R_1, R_2, and R_3 positions of the glyceride backbone. These molecules and their synthesis are described in the specification, for example, at page 7, lines 5 - 24; page 21 line 4 to page 22, line 16; and page 37, line 24 - page 39, line 16. The acylglycerides are characterized in containing at least approximately 30% t10, c12 octadecadienoic acid, at least approximately 30% c9,t11 octadecadienoic acid, and about less than 1% total of 8,10 octadecadienoic acid, 11,13 octadecadienoic acid and trans-trans octadecadienoic acid at positions R_1, R_2, and R_3, wherein the percentages are peak area percentages as determined by gas chromatography. Methods for obtaining isomerized CLA with the desired isomer profile are described in the specification, for example, at page 5, line 14 - page 6, line 20; page 15, line 1 - page 21, line 11; and page 23, line 15 - page 37, line 27. The invention is also directed to food products containing such acylglycerides. See, for example, page 23, lines 1 - 11.

VI. ISSUES

There are three issues involved in the present appeal:

Issue 1 – Whether Claims 5 - 8 are anticipated by Cain et al. (WO97/18320).

Issue 2 - Whether Claims 13 - 17 are obvious over Cain et al. (WO97/18320).

Issue 3 - Whether Claims 5 - 8 and 13 - 17 are obvious over Nilsen et al. (U.S. Pat. No. 5,885,594) in view of Cain et al. (WO97/18320), further in view of Pariza et al. (U.S. Pat. No.

5,017,614).

VII. GROUPING OF CLAIMS

Claims 5 - 8 and 13 - 17 stand or fall together. The remainder of the Claims have separate and distinct limitations and must be considered independently.

VIII. ARGUMENT

A. Issue 1 - Claims 5-8 Are Not Anticipated by Cain *et al.* (WO97/18320)

Claims 5-8 stand rejected under 35 U.S.C. §102 as allegedly being anticipated by Cain *et al.* (WO97/18320). The unrebutted evidence on record conclusively establishes that Cain et al. does not anticipate Claim 5 - 8 because the methods utilized by Cain et al. cannot produce the claimed CLA isomer profile (i.e., a CLA composition containing less than 1% total of 8,10 octadecadienoic acid, 11,13 octadecadienoic acid and trans-trans octadecadienoic acid isomers). The Office has failed to properly consider this evidence.

In response to the rejection over Cain et al., Applicants submitted a Declaration from Asgeir Sæbo (copy attached hereto as Appendix B for the Office's convenience) that establishes that the compositions of Cain et al. necessarily included the 8,10 and 11,13 isomers of CLA. In response to the Sæbo Declaration, the Examiner stated the following:

> In response to applicants' arguments that Mr. Sæbo's experiments show that fatty acid composition obtained by Cain would comprise significant amount of 8,10; 11,13 and other isomers, note Applicant generated data, proffered to obviate prior art teachings, lacks the probative force accorded data generated by independent, disinterested parties. It is well settled patent law "that it is not a difficult matter to carry out a process in such a fashion that it will not be successful and, therefore, the failures of experimenters who have no interest in succeeding should not be accorded great weight."

> In re Michalek, 74 USPQ 108, at 109 citing Bullard Company et al v Coe, 147 F.2d 568, 64 USPQ 359.

Paper 26, pages 3 - 4. For the following reasons, Applicants respectfully submit that the Examiner's reliance on this standard is unfounded.

First, Applicants respectfully submit that the Office's "well-settled" case law is not settled at all, and, in fact, is not in accordance with proper PTO practice. The decision relied on by the Office is more than 50 years old. While this case has not been directly overruled, it is in conflict with current case law and PTO practice. In particular, the Office's failure to accord the proper weight to the Sæbo Declaration does not conform with proper patent practice according the Manual of Patent Examining Procedure (MPEP). The Examiner must respond to all of the arguments and evidence presented by Applicants. The MPEP states that:

> **Office personnel should consider all rebuttal arguments and evidence presented by applicants**. . . . *In re Beattie*, 974 F.2d 1309, 1313, 24 USPQ2d 1040, 1042-43 (Fed. Cir. 1992). . . . **Office personnel should avoid giving evidence no weight**, except in rare circumstances. *Id. See also In re Alton*, 76 F.3d 1168, 1174-75, 37 USPQ2d 1578, 1582-83 (Fed. Cir. 1996).
>
> * * *
>
> A determination under 35 U.S.C. 103 should rest on **all the evidence** and should not be influenced by any earlier conclusion. *See, e.g., Piasecki*, 745 F.2d at 1472-73, 223 USPQ at 788; *In re Eli Lilly & Co.*, 902 F.2d 943, 945, 14 USPQ2d 1741, 1743 (Fed. Cir. 1990). Thus, once the applicant has presented rebuttal evidence, Office personnel should **reconsider** any initial obviousness determination in view of the entire record. *See, e.g., Piasecki*, 745 F.2d at 1472, 223 USPQ at 788; *Eli Lilly*, 902 F.2d at 945, 14 USPQ2d at 1743.[1]

Additionally, the Courts have held as follows:

> When *prima facie* obviousness is established and evidence is submitted in rebuttal, the

[1] MPEP §§2144.08; emphasis added).

> decision-maker must start over . . . . An earlier decision should not . . . be considered as set in concrete, and applicant's rebuttal evidence then be evaluated only its knockdown ability. Analytical fixation on an earlier decision can tend to provide the decision with an undeservedly broadened umbrella effect. *Prima facie* obviousness is a legal conclusion, not a fact. Facts established by rebuttal evidence must be evaluated along with the facts on which the earlier conclusion was reached, not against the conclusion itself. Though the tribunal must begin anew, a final finding of obviousness may of course be reached, but such finding will rest upon evaluation of all facts in evidence, uninfluenced by any earlier conclusion reached . . . upon a different record.[2]

Furthermore:

> If a *prima facie* case is made in the first instance, and if the applicant comes forward with a reasonable rebuttal, **whether buttressed by experiment**, prior art references, or argument, the entire merits of the matter are to be reweighed. (Emphasis added)[3]

Accordingly, even if the Office had established anticipation or a *prima facie* case of obviousness (and Applicants contend that the Office did not), the Office must respond to the information presented in the Declaration. The above directions of the court and the PTO state that the evidence **must be considered**. These directions do not categorize evidence according to whether it is developed by interested or disinterested parties and do not state that evidence developed by the inventor **may be ignored. The directions specifically state that experimental evidence, such as that contained in the Declaration, must be considered.** Indeed, the Examiner must start over and reconsider the entire anticipation or obviousness analysis.

In the present case, there was no reweighing of the merits by the Examiner. Instead of actually analyzing the Declaration and the factual, experimental data contained within it, the

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2 *In re Rinehart*, 531 F.2d 1048, 1052, 189 USPQ 143, 147 (CCPA 1976).

3 *In re Hedges*, 783 F.2d 1038, 1039, 228 USPQ 685, 686 (Fed. Cir. 1986).

Office has summarily dismissed the data with no analysis because it was generated by the Applicants. Applicants submit that the results would be the same no matter who conducts the experiments. The Office has failed to substantively address the data in the Declaration and offer any analysis of why the data is flawed.

Moreover, the holding of *In re Michalek* is factually distinguishable from the present facts of the present application. The Office has attempted to ignore the evidence provided by Applicants by relying on *In re Michalek* and characterizing the evidence as a failure. To the contrary, the evidence presented by the Applicants is not a failure to repeat the results of Cain et al. Cain et al. is **silent** as to the presence of the 8,10 and 11,13 isomers. The only way the Applicants results could be considered to be a failure is if Cain et al. affirmatively stated that the isomers were not present and then Applicants failed not to produce the isomers. This is not the present situation. Applicants results supplement the teachings of Cain, and do not contradict them.

In particular, the Applicants followed the exact instructions of Cain and analyzed the product. The Applicants did not fail to obtain CLA. Indeed, they obtained CLA with the isomers described by Cain et al. However, the fact remains that the CLA also contained other isomers that are not mentioned by Cain. The Examiner apparently places weight on the fact Cain is silent as to the presence of other isomers:

> "Cain et al. do not expressly teach what the remain 36.2 percent of the fatty composition are. However, nowhere in Cain states that "conjugated linoleic acid" are exclusively for c9,t11; and t10,c12 isomers. Any new definition of "CLA" would be improper."

Paper 26, page 3. This statement along with the statement regarding Applicant's improper interpretation of Cain et al. is misplaced and fails to rebut the evidence in the Declaration. Cain

et al.'s silence concerning the presence of the isomers cannot be equated with the absence of the isomers. Indeed, the Examiner proposes a definition of "conjugated linoleic acid" that is found nowhere in Cain et al. In particular, Cain et al. does not specifically define CLA to include non-active CLA isomers. As noted by Mr. Saebo, the results presented in Cain:

> [d]o not mean that the other isomers were not present, as was found in my repeat of Cain. This discrepancy is explainable by the facts that 1) methods for the analysis of CLA compositions in 1996 were rather crude and 2) Cain may have simply chosen not to include non-active isomers when reporting their results. Improved methods for detecting the various isomers of CLA were not developed until well after the 1995 priority date of Cain. This fact is substantiated by Yurawecz *et al.* (attached at Tab 2), who state "the CLA products analyzed in this study were found to contain up to 12 geometric and positional CLA isomers. These findings are based on appropriate and improved analytical methodologies [including gas chromatography techniques] that have only recently been developed." (Yurawecz, *p.* 281). Thus, Cain et al. may not have conducted an analysis which could detect the isomers in questions. Consideration of Example 18 of Cain et al. supports this analysis. The inventors state that their compositions, produced by the method of Example 6, contained 63.8% CLA, of which 48.9% was the cis 9, trans 10 isomer and 51.1% was the trans 10, cis 12 isomer. This means that the inventors provide no analysis of the remaining 36.2% of their composition. The 8,10; 11,13; and trans-trans isomers that are discriminated against in the present invention and detected in my repeat of Cain could well have been present in this fraction.

Thus, the Examiner's conclusions regarding Cain et al. are rebutted by actual experimental evidence.

Additionally, other evidence not generated by the Applicants is consistent with the Applicants data. No chemical isomerization method has been described in the literature to date which does not produce a variety of isomers. Indeed, it is impossible to isomerize linoleic acid by the methods described in Cain without producing other isomers due to the process known as thermal sigmatropic rearrangement. This process is described in Chapter 5 of the book *Advances*

in Conjugated Linoleic Acid Research, Volume 2, J. Sebedio, W.W. Christie, and R. Adolf, Eds., AOCS Press, Champaign, IL, 2002 (Attached hereto as Appendix C). Mr. Saebo wrote this chapter. Briefly, the research described in this chapter establishes that the formation of the 8,10 and 11,13 isomers is a necessary consequence of heating compositions containing the t10,c12 and c9,t11 isomers. Thus, whenever compositions containing t10,c12 and c9,t11 CLA are heated at temperatures such as those used by Cain et al. (i.e., 180°C for about 2 - 2.5 hours), 8,10 and 11,13 isomers are necessarily produced. Accordingly, it appears that Cain et al. simply failed to conduct an analysis for the other isomers present in the isomerized product since those isomers were necessarily present.

This conclusion is also supported by reference to Sugano et al., Lipids 33(5):521-527 (1998)(reference 47 in Form 1449 filed April 10, 2000, copy attached hereto at Appendix D for the Office's convenience). Sugano et al. isomerized linoleic acid conditions similar to those described by Cain et al. The conditions utilized in the two references are compared in following Table.

Cain et al.	**Sugano et al.**
50 g linoleic acid, 95% pure	50 g linoleic acid, 99% pure
Solvent: 290 grams ethylene glycol	Solvent: 290 grams ethylene glycol
Catalyst: 15 g NaOH	Catalyst: 15 g NaOH
Reaction time: 2 hours	Reaction time: 2 hours
Reaction temperature: 180° C	Reaction temperature: 180° C
Reaction atmosphere: Inert	Reaction atmosphere: Nitrogen

As can be seen, the reaction conditions were almost identical. However, the results are not. As

noted on page 522 of Sugano, the resulting CLA preparation contained the following CLA isomers: 29.8% c9,t11/t9,c11; 29.6% t10,c12; 1.3% c9,c11; 1.4% c10,c12; 18.6% t9,t11/t10,t12; 5.6% linoleic acid; and 13.7% other isomers. In contrast to the simplified analysis presented in Cain et al., isomerization of CLA results in the production of many different isomers, not just the desired c9,t11 and t10,c12 isomers.

As further support for this fact, the Board's attention is respectfully directed to examples 1-4 of the instant application. These examples compare non-aqueous alkali isomerization under high or low temperatures and aqueous alkali isomerization under high or low temperatures. **The important fact to note is that each reaction, even the low temperature non-aqueous alkali isomerization reaction (Example 1, Table 6), produced a distribution of the expected isomers, not just the c9,t11 and t10,c12 isomers.** Thus, ample evidence supports the conclusion that the analysis of Cain et al. is either incorrect or incomplete. Cain et al. appear to have either not analyzed for the isomers or chosen not to present data pertaining to the other isomers in their analysis. This is understandable because at the time, the other isomers were not expected to have a biological effect. However, just because data on these isomers was not presented does not mean that they are not present. Indeed, the evidence establishes that they were necessarily present as a result of the reaction conditions used by Cain et al. **Thus, the compositions of Cain necessarily contained levels 8,10; 11,13; and trans,trans isomers that do not meet the claimed levels.** Cain et al. does not anticipate the claims.

Additionally, to the extent that the Examiner is relying on anticipation by inherency, that reliance is misplaced. The Federal Circuit has stated that inherency cannot be based upon information provided in the disclosed invention. Indeed, to establish inherency the prior art must have recognized at the time of the Applicant's invention, that the elements now at issue were

inherently present in the Examiner's reference. For example, in *Continental Can Co. USA v. Monsanto Co.*, the Federal Circuit held that:

> To serve as an anticipation when the reference is silent about the asserted inherent characteristic, such gap in the reference may be filled with recourse to extrinsic evidence. Such evidence must make clear that the missing descriptive matter is necessarily present in the thing described in the reference and that it would be so recognized by persons of ordinary skill.

(*Continental Can Co. USA v. Monsanto Co.*, 212 USPQ 323 [CCPA 1981]; emphasis added). Furthermore, "[i]n relying upon the theory of inherence, the examiner must provide a basis in fact/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art." (MPEP §2112, quoting, *In re Robertson*, 169 F.3d 743 [Fed. Cir. 1999]; emphasis original). Applicants again submit, that since the prior art at the time of Applicants' invention did not recognize compositions or methods of producing compositions comprising less than 1% of particular CLA isomers that the cited reference does not anticipate the presently claimed invention.

Accordingly, Applicants respectfully request that this ground of rejection be removed and the claims passed to allowance.

B. Issue 2 - Claims 13-17 Are Not Obvious Over WO97/18320

Claims 13-17 stand rejected under 35 U.S.C. §103 as allegedly being obvious over Cain *et al.* (WO97/18320). Applicants respectfully note that a *prima facie* case of obvious requires that all elements of the claims be present in the cited reference. As established above, the compositions of Cain necessarily contained levels 8,10; 11,13; and trans,trans isomers that do not meet the claimed levels. Thus, Cain et al. does not render the claims obvious. Accordingly, Applicants respectfully request that this ground of rejection be removed and the claims passed to

allowance.

C. Issue 3 - Claims 5-8 and 13-17 Are Not Obvious Over the Combination of Nilsen, Cain and Pariza

Claims 5-8 and 13-17 stand rejected under 35 U.S.C. §103(a) as allegedly being obvious under Nilsen *et al.* (U.S. Pat. No. 5,885,594) in view of Cain *et al.* (WO97/18320), further in view of Pariza *et al.* (US Pat. No. 5,017,614). The Office has failed to establish a *prima facie* case of obviousness because the references, alone or in combination, fail to teach each element of the claimed compositions. In particular, the combined references do not teach acylglyceride compositions comprising less than 1% 8,10; 11,13; and trans-trans isomers and containing at least approximately 30% t10,c12 and 30% c9,t11 isomers.

Applicants first note that as discussed in detail above, Cain et al. does not teach compositions comprising less than 1% 8,10; 11,13; and trans-trans isomers or methods of obtaining such compositions.

Likewise, Nilsen et al. provides no such compositions or methods. Indeed, Nilsen et al. **do not teach any method** at all for conjugation, they merely list CLA in a long list of fatty acids that may be useful in their invention. This fact is verified in paragraph 6 of the Saebo Declaration.

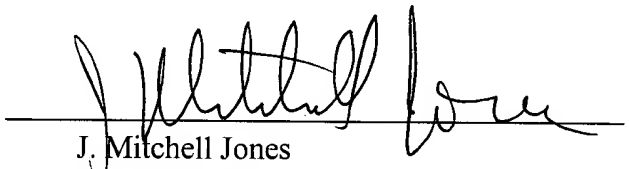
Furthermore, as discussed in Paragraph 7, Pariza et al. further fails to cure this deficiency. In particular, Pariza does not teach preparation of CLA in amounts suitable for incorporation into acylglycerides. Indeed, the HPLC purified isomers are produced for use as chromatography standards. Importantly, because the isomers are produced for use as standards, Pariza does not teach or suggest combining the isomers to form a composition containing both t10,c12 and t9,c11

isomers as required by the Claims. Thus, the passage cited by the Office (column 4, line 50 - bridging column 5, line 68) actually teaches away from a combination of isomers as required by the Claims. The Office is respectfully reminded that references that teach away cannot provide a motivation to combine or modify. *See Tec Air, Inc. v. Denso Manufacturing Michigan, Inc.*, 192 F.3d 1353. Accordingly, Applicants respectfully request that this ground of rejection be removed and the claims passed to allowance.

D. Conclusion

For the foregoing reasons, it is submitted that the Office's rejection of Claims 5 - 8 and 13 -1 7 was erroneous, and reversal of the rejection is respectfully requested. Appellant requests either that the Board render a decision as to the allowability of the claims, or alternatively, that the application be remanded for reconsideration by the Office.

Dated: November 25, 2003



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APPENDIX A

CLEAN VERSION OF THE ENTIRE SET OF PENDING CLAIMS

5. (Amended three times) A biologically active acylglycerol composition comprising a plurality of acylglycerol molecules wherein the acylglycerol molecules comprise substituents R_1, R_2, and R_3 attached at the positions of the OH- groups of a glycerol backbone, and wherein R_1, R_2, and R_3 are selected from the group consisting of a hydroxyl group and an octadecadienoic acid, said composition characterized in containing at least approximately 30% t10,c12 octadecadienoic acid, at least approximately 30% c9,t11 octadecadienoic acid, and about less than 1% total of 8,10 octadecadienoic acid, 11,13 octadecadienoic acid and trans-trans octadecadienoic acid at positions R_1, R_2, and R_3, wherein said percentages are peak area percentages as determined by gas chromatography.

6. The composition of claim 5, further comprising a food product incorporating said acylglycerol composition.

7. The composition of claim 6, wherein said food product is for human consumption.

8. The composition of claim 6, wherein said food product is a feed formulated for animal consumption.

13. (Amended three times) A composition comprising a prepared food product containing a biologically active acylglycerol composition comprising a plurality of acylglycerol molecules wherein the acylglycerol molecules comprise substituents R_1, R_2, and R_3 attached at the positions of the OH- groups of a glycerol backbone, and wherein R_1, R_2, and R_3 are selected from the group consisting of a hydroxyl group and an octadecadienoic acid, said composition characterized in containing at least approximately 30% t10,c12 octadecadienoic acid, at least approximately 30% c9,t11 octadecadienoic acid, and about less than 1% total of 8,10 octadecadienoic acid, 11,13 octadecadienoic acid and trans-trans octadecadienoic acid at

15. The composition of Claim 13, wherein said prepared food product is a drink.

16. The composition of Claim 13, wherein said prepared food product is a snack food.

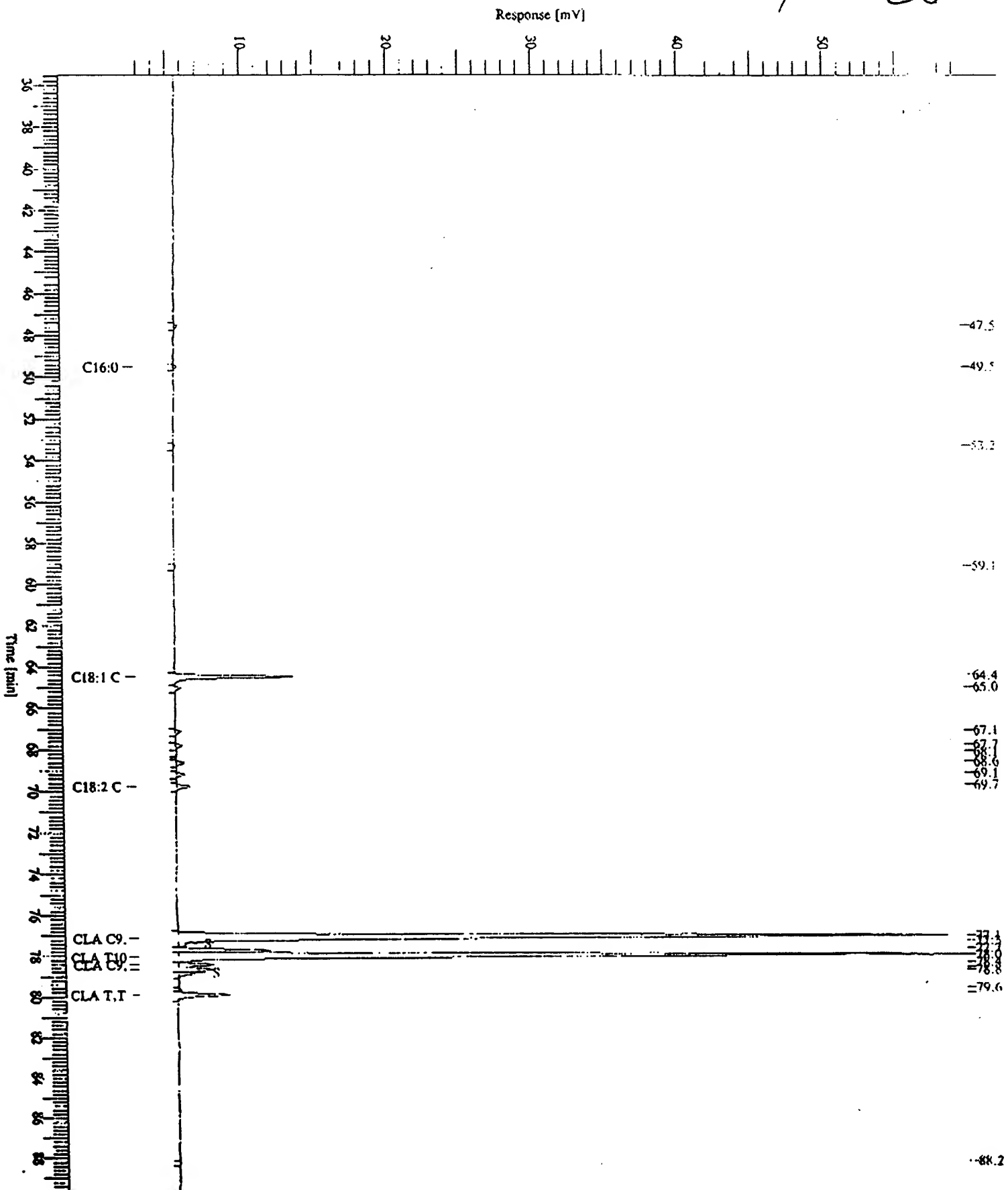
17. The composition of Claim 13, wherein said prepared food product is a frozen meal.

APPENDIX B

Declaration of Asgeir Saebo with attachments

Sample Name : 6659: A01348, 024/96-1, CLA PPA Sample #: 001 Page 1 of 1
FileName : D:\TCWS Data\data\Data 100E 1000-15_,_\100e1001.raw
Date : 19.11.01 11:48:38
Method : Time of Injection: 19.11.01 09:08:32
Start Time : 35.53 min End Time : 89.83 min Low Point : 2.75 mV High Point : 59.51 mV
Plot Offset: 2.75 mV Plot Scale: 56.8 mV

WO 97/18320



Software Version : 6.1.2.0.1:D19
Sample Name : 6659: A01348, 024/96-1, CLA FFA
Instrument Name : GC
Rack/Vial : 0/1
Sample Amount : 1.000000
Cycle : 1
Date : 19.11.01 11:48:37
Data Acquisition Time : 19.11.01 09:08:32
Channel : B
Operator : Operatør
Dilution Factor : 1.000000

Result File : D:\TCWS Data\data\Data 100E 1000-1999\100e1001.rst
Sequence File : D:\TCWS Data\sekvenser\100E.20.10.00..seq

FATTY ACID PROFILE REPORT

PERKIN ELMER AUTOSYSTEM XL GC

Column: WCOT FUSED SILICA 100 m x 0.25 mm COATING CP-SIL 88 DF= 0.2 Chrompack cat.no: 7489
Carrier Gas: He, 30.0 PSI
Method: 100E.mth
Temp: 80 C (2 min)->45 C/ min->130 C (0 min)->1 C/ min-> 220 C (10 min)
Injection: Splitless, 240 C
Detector: FID, 280 C

Peak #	Time [min]	Component Name	Area [%]	Area [µV·s]	Height [µV]
1	47.557		0.14	2040.57	221.66
2	49.507	C16:0	0.12	1770.08	234.26
3	53.277		0.07	1043.10	118.41
4	59.139		0.07	1079.52	131.55
5	64.461	C18:1 c9	4.84	72109.91	8053.81
6	65.035		0.23	3435.33	396.61
7	67.125		0.25	3718.15	401.86
8	67.795		0.28	4195.57	459.60
10	68.621		0.31	4688.64	520.82
11	69.176		0.33	4880.16	532.98
12	69.744	C18:2 c9,c12	0.53	7977.36	868.60
13	77.128	CLA c9,t11+t8,c10	42.84	638739.60	52812.75
14	77.371		0.28	4120.52	216.07
15	77.752	CLA c11,t13	3.49	51987.22	6233.41
16	78.067	CLA t10,c12	40.35	601682.23	54289.00
17	78.437	CLA c9,c11	1.36	20327.77	2373.19
18	78.664	CLA c10,c12	1.61	24007.50	2280.68
19	78.808		0.58	8661.37	1107.38
20	79.693		0.08	1265.48	173.63
21	79.909	CLA t,t 9,11+10,12	2.24	33420.59	3512.11
			100.00	1491150.67	134938.38

Missing Component Report

Component	Expected Retention (Calibration File)
C18:0	0.001

19.11.01 11:48:37 Result: D:\T\ /S Data\data\Data 100E 1000-1999\100e1001.rst

Analyzed by: Natural ASA, Hovdebygda

Approved by: _____

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Contents

Variations in isomer distribution in commercially available conjugated linoleic acid*

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Conjugated linoleic acid (CLA) has been reported to have anticarcinogenic and antiatherogenic properties, to repartition body fat, to build bone mass, to normalize glucose tolerance, and to reduce hyperglycemia and diabetes. CLA products are now commercially available, and there is considerable interest in studying CLA because of this range of reported beneficial effects. However, little is known about the composition of these preparations. Representative commercial CLA products in capsule or liquid (aqueous or oily) form were analyzed for their CLA content and isomer composition using gas chromatography (GC), silver ion-high performance liquid chromatography (Ag^+-HPLC) and spectroscopic techniques. The content of CLA in the preparations varied widely. Based on the GC-internal standard technique, total CLA varied from 20 to 89% by total weight and 28 to 94% of total fat. One product contained no CLA. The isomer distributions were generally of two types: those with two major CLA positional isomers, and those with four major CLA positional isomers. All the CLA preparations in capsule form contained the four isomer mixture, while the liquid preparations contained from two to four CLA positional isomers.

Unterschiede in der Isomerenverteilung kommerziell erhältlicher konjugierter Linolsäure. Von konjugierter Linolsäure (CLA) wurde berichtet, daß sie anticanzerogene und antiatherogene Eigenschaften hat, Körperfett repartioniert, Knochenmasse aufbaut, Glukosetoleranz normalisiert und Hyperglykämie und Diabetes reduziert. CLA-Produkte sind jetzt kommerziell erhältlich, und es gibt wegen der oben aufgeführten prositiven Effekte ein beträchtliches Interesse daran, CLA zu studieren. Allerdings ist wenig bekannt über die Zusammensetzung dieser Herstellungen. Repräsentative kommerzielle CLA-Produkte in Kapsel- oder flüssiger Form (auf Wasser- oder Ölbasis) wurden mit Hilfe eines Gaschromatographen (GC), Silberionen-Hochdruckflüssigkeitschromatographie (Ag^+-HPLC) und spektroskopischer Techniken auf ihren CLA-Inhalt und ihre isomere Zusammensetzung analysiert. Der Inhalt der CLA in den Herstellungen variierte stark. Auf der Basis der GC-internen Standardtechnik schwankten die gesamten CLA zwischen 20 und 89% bezogen auf das Gesamtgewicht und zwischen 28 und 94% bezogen auf den Gesamtfettanteil. Ein Produkt enthielt keine CLA. Die Isomerverteilungen untergliederten sich allgemein in zwei Typen: solche mit zwei Positionsisomeren und solche mit vier Positionsisomeren. Alle CLA-Herstellungen in Kapselform beinhalteten das Gemisch der vier Isomere, während die flüssigen Herstellungen zwischen zwei und vier der Positionsisomere enthielten.

1 Introduction

Conjugated linoleic acid (CLA) has been reported to provide direct [1] or indirect [2] protection against several types of cancer, atherosclerosis [3, 4], and diabetes [5]. CLA has also been reported to improve feed efficiency [6] and increase muscle [7, 8], and bone mass [9]. These results were generally obtained in experimental animals fed commercial CLA preparations containing approximately equal amounts of four *cis/trans* conjugated octadecadienoic (18:2) acids: 8 *trans*, 10 *cis*-18:2; 9 *cis*, 11 *trans*-18:2; 10 *trans*, 12 *cis*-18:2; 11 *cis*, 13 *trans*-18:2; and minor amounts of the corresponding *cis,cis* and *trans,trans* CLA isomers [10]. Thus, the contribution(s) of the specific isomers to the observed effects are not known. In contrast, natural products, such as milk, cheese, and meat from ruminant animals contain mainly rumenic acid (9 *cis*, 11 *trans*-18:2) [11–13] with minor amounts of 7 *trans*, 9 *cis*-18:2 [14] and other isomers [15–17]. The total CLA content in these natural products ranges from trace to 2% of total fatty acids [12, 18, 19].

The present study was undertaken to determine the content and distribution of CLA isomers in commercially available CLA capsules and liquid products with labels stating to contain CLA. The CLA isomers were analyzed by gas chromatography (GC) and silver ion-high performance liquid chromatography (Ag^+-HPLC) as their fatty acid methyl esters (FAME), and identified by GC-electron ionization mass spectroscopy (GC-EIMS) and GC-direct deposition-Fourier transform infrared (GC-DD-FTIR) spectroscopy as their 4,4-dimethyloxazoline derivatives [10, 14, 17, 20].

2 Materials and Methods

2.1 Chemicals

Representative CLA preparations were purchased locally, from specialty chemical companies, or from the World Wide Web. Several pure CLA isomers were obtained as their free fatty acids from *Matreya*, Inc. (Pleasant Gap, PA). Acetonitrile and hexane were UV grade. Other solvents were distilled-in-glass quality. 2-Amino-2-methyl-1-propanol (95%) was purchased from *Aldrich Chemical Company*, Inc. (Milwaukee, WI). A 10% solution of trimethylsilyldiazomethane in hexane was obtained from *TCI America* (Portland, OR).

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Anhydrous $NaOCH_3$/methanol was purchased from *Supelco*, Inc. (Bellefonte, PA.).

2.2 Lipid extraction

A known weight (approximately 25 mg) of each product was dissolved in 2 ml 1 N KOH in ethanol (95%) and hydrolyzed overnight in the dark at room temperature. For quantitative analyses, one mg of eicosanoic acid (23:0) was added as an internal standard. After hydrolysis, 5 ml of H_2O and one ml of 6 N HCl were added and the free fatty acids were extracted three times with 5 ml diethyl ether/petroleum ether (1:1). The combined extracts were washed with H_2O and dried over anhydrous Na_2SO_4, and the solvents removed under a stream of argon. Aqueous CLA samples were first extracted with petroleum ether/diethyl ether (1:1), and 25 mg of the extracted lipids were treated as described above.

2.3 Derivatizations

FAMEs were prepared for GC by dissolving the free fatty acids in one ml of benzene/methanol (4:1) to which 0.5 ml of a 10% solution of trimethylsilyldiazomethane in hexane were added [21]. The reaction was allowed to stand for 0.5 h with occasional gentle shaking. Thereafter, five drops of glacial acetic acid were added with gentle shaking. The same amount of glacial acetic acid was added to each of the solutions to destroy excess yellow trimethylsilyldiazomethane. Some solutions did not become clear on addition of glacial acetic acid. Then 5 ml of H_2O were added, and the reaction mixture was extracted with one ml of isooctane. The extract was subsequently dried over anhydrous Na_2SO_4.

The 4,4-dimethyloxazoline (DMOX) derivatives were prepared to determine the double bond position of CLA isomers. Ten to 20 mg of the free fatty acid product prepared above was added to a screw cap reaction tube (1 ml) and a threefold excess (w/w) of 2-amino-2-methyl-1-propanol was added. The tube was purged with argon, capped, and heated at 170 °C for 0.5 h in an oven. DMOX derivatives were then partitioned into petroleum ether as described previously [22].

2.4 Gas chromatography

The analyses of the FAMEs were carried out using a *Hewlett-Packard* (Palo Alto, CA) model 5890 gas chromatograph fitted with a flame-ionization detector. A CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm i.d. × 0.2 µm film thickness; *Chrompack*, Bridgewater, NJ) was used, and H_2 was the carrier gas at a split ratio of 50:1. The column was operated at 75 °C for 2 min, then temperature-programmed at 5 °C/min to 185 °C, held for 30 min, followed by a second temperature program at 4 °C/min to 225 °C and held there for 33 min.

2.5 Ag^+-HPLC

The HPLC (*Waters 510* solvent delivery system; *Waters Associates*, Milford, MA) was equipped with an autosampler and 200-µl injection loops (*Waters 717*), a UV detector operated at 233 nm (*Waters 486* tunable absorbance), and a data system (*Waters Millennium*™ version 2.15). A *ChromSpher 5 Lipids* analytical silver impregnated column (4.6 mm i.d. × 250 mm stainless steel; 5 µm particle size; *Chrompack*, Bridgewater, NJ) was operated at room temperature. The mobile phase was 0.1% acetonitrile in hexane and operated isocratically at a flow rate of 1.0 ml/min. The retention times varied slightly between runs due to the insolubility of acetonitrile in hexane. However, these changes did not affect the relative elution sequence of CLA isomers. Typical injection volumes were 5–15 µl at a concentration of 1 mg total FAME per ml.

2.6 Gas chromatography – electron ionization mass spectrometry

The GC-EIMS analyses were performed by using a *Hewlett-Packard* (model 5890, series II) GC coupled to a mass spectrometer (*Autospec Q* mass spectrometer) and a data system (*OPUS 4000; Micromass*, Manchester, UK). The GC-EIMS system utilized version 2.1 BX software. This system was used with a 50 or 100 m CP-Sil 88 fused-silica capillary column. The GC-EIMS conditions were: splitless injection with helium or hydrogen as the carrier gas and sweep was restored 1 min after injection. The injector and transfer lines temperatures were 220 °C. The column was operated at 75 °C for one min after injection, then temperature-programmed 20 °C/min to 185 °C, held there for 15 min, then temperature-programmed 4 °C/min to 220 °C, and held there for 45 min.

Tab. 1. Conjugated linoleic acid (CLA) methyl ester isomers, as % of total CLA, in 13 commercial CLA preparations as determined by silver ion-high performance liquid chromatography (Ag^+-HPLC).

Product	*trans,trans*				*cis/trans*[a]				*cis,cis*			
	11,13	10,12	9,11	8,10	11,13	10,12	9,11	8,10	11,13	10,12	9,11	8,10
1 aqueous	0[b]	0	0	0	0	0	0	0	0	0	0	0
2 oil	0	1.1	1.0	tr[c]	0	47.1	50.8	tr	0	tr	tr	0
3 oil	0	0.5	0.5	0	1.1	50.2	47.6	tr	0	tr	tr	0
4 oil	0	1.1	1.3	0	0	45.8	50.7	tr	0	1.1	0.1	tr
5 oil	0	0.6	0.6	0	0	54.0	43.5	0	0	0.7	0.6	0
6 oil	1.5	5.4	11.9	7.1	2.2	38.3	21.7	tr	0	2.3	6.2	3.5
7 capsule	0.7	2.7	2.8	0.5	19.0	32.1	25.6	15.6	0.7	0.4	tr	tr
8 capsule	0.8	2.7	2.5	0.4	16.8	33.9	27.1	14.2	tr	1.7	tr	tr
9 capsule	1.0	3.1	2.8	0.5	16.9	33.7	26.9	14.0	0.6	0.5	tr	tr
10 capsule	0.7	2.5	2.5	0.6	15.5	31.0	27.7	14.2	0.8	2.2	1.8	0.6
11 capsule	0.4	2.8	2.9	0.5	14.4	29.1	30.0	15.9	0.5	1.5	2.1	tr
12 capsule	1.3	3.3	3.4	1.1	19.8	25.9	21.6	16.5	1.2	2.7	2.4	1.0
13 oil	4.0	5.4	5.4	1.7	19.7	26.8	25.6	10.5	0.2	0.1	0.6	0.1

[a] The CLA isomers exist either in the *cis,trans* or *trans,cis* configuration. [b] 0, not detectable. [c] tr, trace (<0.05%).

2.7 GC-direct dep sition Fourier transform infrared spectroscopy

GC-DD-FTIR was performed using a *Bio-Rad* (Cambridge, MA) *Tracer™ GC-FTIR 60A* spectrometer system. This system was used with a 50 m CP-Sil-88 fused-silica capillary column as described previously [23, 24].

3 Results and Discussion

Preparations of CLA were capsules or liquids that were water-based or oil-based; some contained non-lipid material. Their chemical compositions were not known. Based on the assumption that the CLA products consisted of esters, free fatty acids or combinations thereof, all products were hydrolyzed under alkali conditions and subsequently methylated by using trimethylsilyldiazomethane as catalyst to ensure preservation of the original distribution of CLA isomers [25].

Total fatty acid compositions of the 13 CLA products were determined. The internal standard (23:0) added to the CLA products provided a mean to determine the amount of total fatty acids in the original CLA mixture. When this total fatty acid value was compared to the 25 mg of starting material used, an approximate estimate of the non-lipids in the sample was obtained. The approximate amount of non-lipid material calculated for these products ranged from 3 to 28%. In addition, unidentified FAMEs ranged from 1 to 29%. The

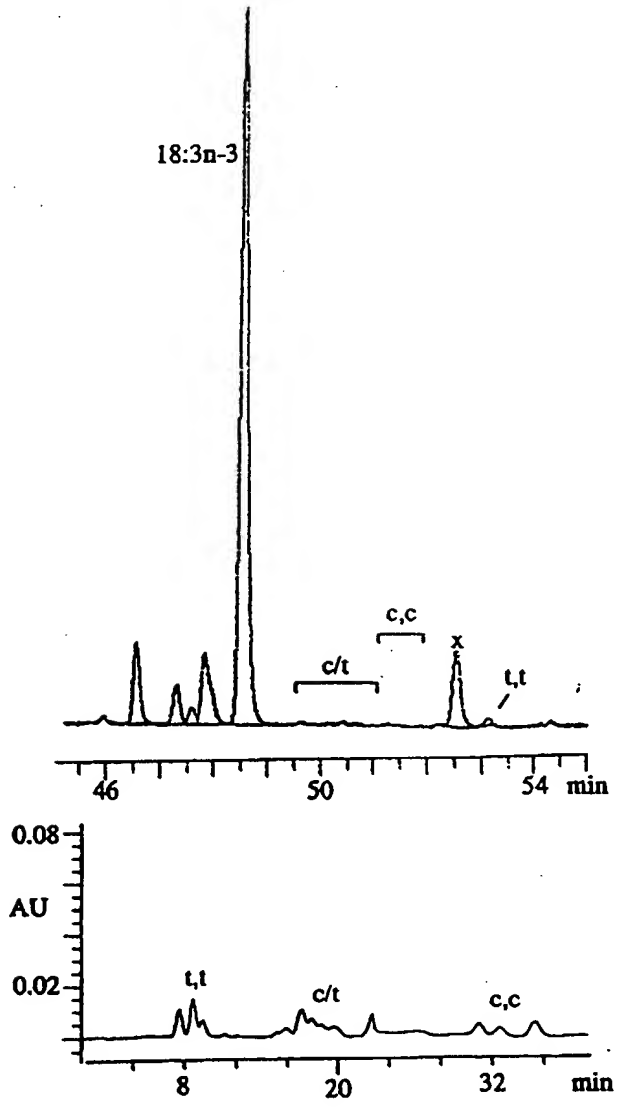

Fig. 1. Gas (upper graph) and silver ion-high performance liquid (lower graph) chromatograms of a commerial conjugated linoleic acid (CLA) preparation containing no CLA. The corresponding CLA regions in each chromatogram are labelled; x is an unknown component. The absorbance scale is shown in the lower graph to indicate the low response found in the CLA region.

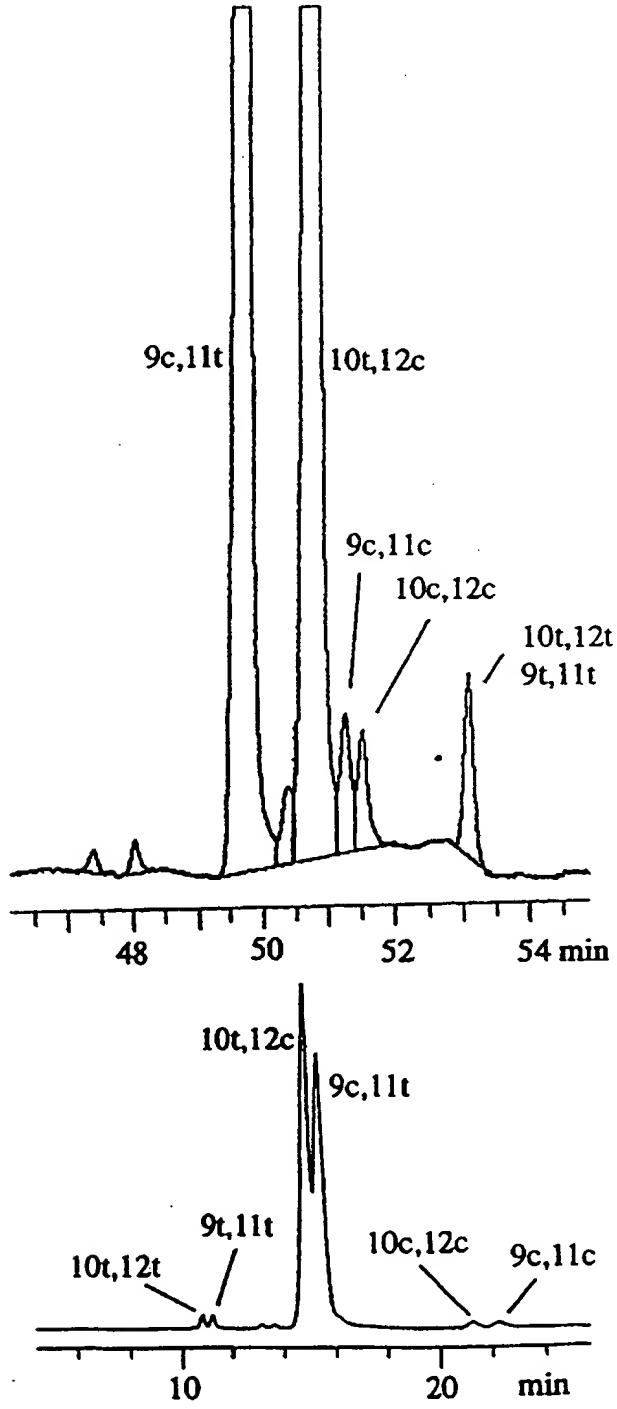

Fig. 2. Gas (upper graph) and silver ion-high performance liquid (lower graph) chromatograms of a representative commerial conjugated linoleic acid (CLA) preparation consisting primarily of two CLA isomers, 9 *cis*, 11 *trans*-18:2 and 10 *trans*, 12 *cis*-18:2. Peaks corresponding to CLA isomers in each chromatogram are labelled.

major fatty acids, other than the CLA isomers found in the preparations, included palmitic (16:0), stearic (18:0), oleic (18:1n-9), and linoleic (18:2n-6) acids. Their combined content ranged from 1 to 84% in the products examined. Based on the GC-internal standard technique, total CLA content ranged from 0 to 94% of the total FAMEs, or 0 to 89% of the mass content of the products.

The CLA isomer distributions in the products, analyzed by a combination of GC and Ag^+-HPLC, are shown in Tab. 1. No CLA was found in product 1. The CLA-containing products fell into two catagories: those composed of two major CLA positional isomers (9 *cis*, 11 *trans*-18:2 and 10 *trans*, 12 *cis*-18:2), and those composed of four major CLA positional isomers (8 *trans*, 10 *cis*-18:2; 9 *cis*, 11 *trans*-18:2; 10 *trans*, 12 *cis*-18:2, and 11 *cis*, 13 *trans*-18:2). Other minor CLA isomers were present at much lower concentrations, but are not reported. All the CLA products in capsule form contained the mixture of four isomers, while the liquid products contained either two or four CLA positional isomers. Representative GC and Ag^+-HPLC chromatograms for these groups are shown in Figs. 1, 2, and 3, respectively.

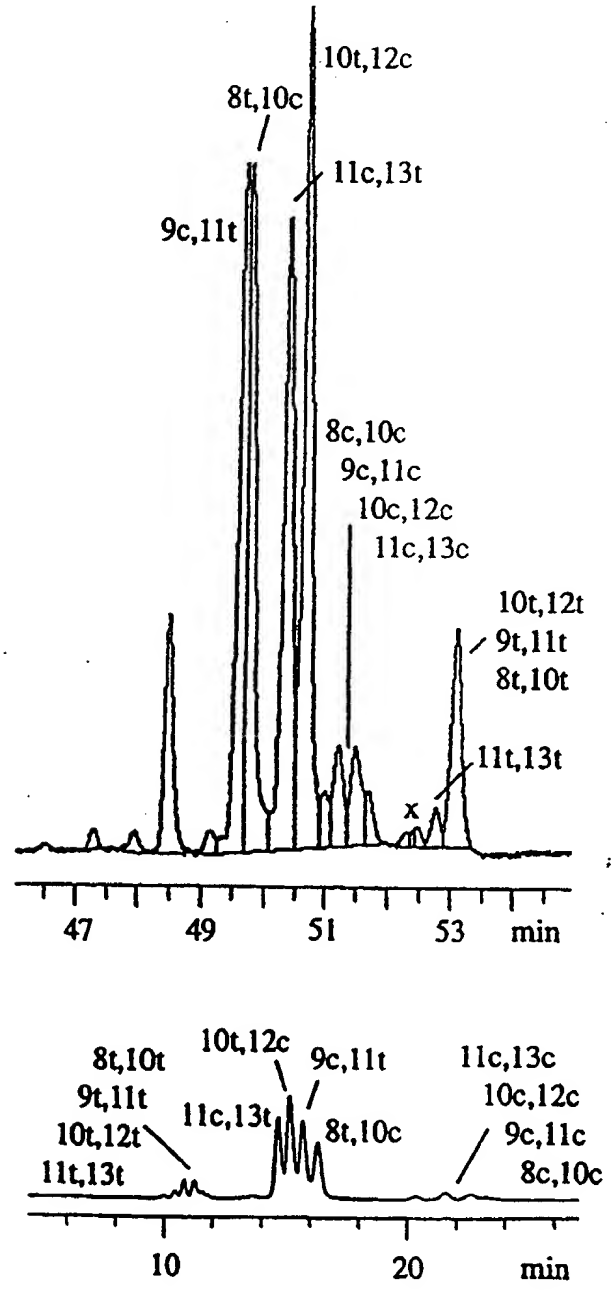

Fig. 3. Gas (upper graph) and silver ion-high performance liquid (lower graph) chromatograms of a representative commerial conjugated linoleic acid (CLA) preparation consisting primarily of four CLA isomers, 8 *trans*, 10 *cis*-18:2, 9 *cis*, 11 *trans*-18:2, 10 *trans*, 12 *cis*-18:2 and 11 *cis*, 13 *trans*-18:2. Peaks corresponding to CLA isomers in each chromatogram are labelled; x is an unknown component.

An explanation for the differences in isomer distributions among the products was not available. Alkali isomerization of 18:2 n-6 in laboratory scale batches prepared according to published procedures [12, 26, 27] resulted in the formation of only two CLA isomers, i.e., 9 *cis*, 11 *trans*-18:2 and 10 *trans*, 12 *cis*-18:2. Alkali isomerization under large-scale, and possibly more severe conditions, may have produced the four CLA positional isomer pattern observed in the commercial CLA preparations described here. This will need to be confirmed.

The GC analyses were based on use of a 100 m polar capillary column. In this system, 8 *trans*, 10 *cis*-18:2 was not resolved from 9 *cis*, 11 *trans*-18:2. A shoulder or a split peak may occasionally be evident when the amounts of these two isomers are approximately equal. In contrast, 11 *cis*, 13 *trans*-18:2 eluted before and was resolved from 10 *trans*, 12 *cis*-18:2 using this GC column (Fig. 3, upper graph). The *cis,cis* CLA isomers eluted after 10 *trans*, 12 *cis*-18:2 in the order 8, 10-, 9, 11-, 10, 12-, and 11,13-18:2 as established previously [10, 20]. The last CLA isomers to elute were the *trans,trans*, consisting of a small peak due to 11,13-18:2 followed by an unresolved mixture of 10,12-, 9,11-, and 8,10-18:2 as demonstrated previously [14, 17]. A small unknown peak was observed between the *cis,cis* and the *trans,trans* CLA isomer regions. The structural identity of all CLA isomers was established and confirmed by analyzing the DMOX derivatives of selective CLA products by GC-EIMS and GC-DD-FTIR. Representative mass and infrared spectra were published previously [10, 14, 17, 27].

Chromatograms showing the separation of the CLA isomers by Ag^+-HPLC are presented below the GC chromatograms in Figs. 1 to 3. The elution orders of all the geometric (in the order *trans,trans*, *cis/trans*, and *cis,cis*) and positional (in the order 11,13-, 10,12-, 9,11-, and 8,10-18:2) CLA isomers by Ag^+-HPLC were established previously [10]. Ag^+-HPLC was essential to complement the GC analysis and establish the composition of 8 *trans*, 10 *cis*-18:2 and 9 *cis*, 11 *trans*-18:2, and the distribution of most of the *trans,trans* CLA isomers.

In contrast to the commercial CLA preparations, that were found to contain two or four CLA positional isomers, natural dairy products and meats from ruminant animals contain primarily rumenic acid, 9 *cis*, 11 *trans*-18:2 [11, 12, 14, 17–19]. While it has not been established, which isomer(s) is (are) responsible for the reported beneficial properties of CLA, it is generally thought that anticarcinogenicity is due to rumenic acid [12, 15]. The nutritional and physiological effects, if any, of other CLA isomer(s) in commercially available CLA preparations are not known.

We recently reported, that ne of the four major *cis/trans* CLA isomers, 11 *cis*, 13 *trans*-18:2, accumulates preferentially in heart phospholipids and specifically in heart and liver diphosphatidylglycerol (DPG) of pigs fed a CLA mixture containing four positional isomers [20]. DPG is a major component of inner mitochondrial membranes and is involved in many enzymes of bioenergetics in the mitochondria [28, 29]. *Watkins* et al. [30] demonstrated that docosahexaenoic acid (22:6 n-3) accumulated in DPG of human colonic adrenocarcinoma (HT-29) cells and increased mito-

chondrial oxidant production. Similarly, 11 *cis*, 13 *trans*-18:2 (or any other CLA isomer incorporated into DPG), could affect mitochondrial oxidant production, particularly since it has been shown that the oxidative susceptibility of CLA is comparable to that of arachidonic acid (20:4 n-6) [31, 32]. In response to our findings that 11 *cis*, 13 *trans*-18:2 was selectively incorporated into DPG [20], a major supplier of commercial CLA preparations recently modified the process to eliminate the 11 *cis*, 13 *trans*-18:2 isomer. The simultaneous elimination of 8 *trans*, 10 *cis*-18:2 from the resulting CLA mixture was an additional benefit of preparing a CLA mixture devoid of 11 *cis*, 13 *trans*-18:2.

In conclusion, the CLA products analyzed in this study were found to contain up to 12 geometric and positional CLA isomers. These findings are based on appropriate and improved analytical methodologies that have only recently been developed [10, 14, 16, 17, 20, 27]. All commercially available CLA products investigated differ, some significantly, and the isomers present may not necessarily represent active CLA components. As new products consisting of two or perhaps only one CLA isomer become available, it will be possible to determine the physiological effects of specific isomers. This is essential for an understanding of this unusual group of lipids.

Abbreviations

Ag^+-HPLC, high-performance liquid chromatography; *cis/trans*, refers to all the CLA isomers having either a *cis,trans* or a *trans,cis* configuration; CLA, conjugated linoleic acid; DMOX, 4,4-dimethyloxazoline; FAME, fatty acid methyl esters; GC-DD-FTIR, gas chromatography-direct deposition-Fourier transform infrared; GC-EIMS, gas chromatography-electron ionization mass spectrometry.

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Changes in Conjugated Linoleic Acid Composition Within Samples Obtained from a Single Source

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ABSTRACT: Conjugated linoleic acid (CLA; 9*c*,11*t*-18:2) and CLA isomers have been reported, in animals, to exhibit a variety of health-related benefits. Silver ion high-performance liquid chromatography (Ag-HPLC) was found to provide better resolution of the isomers than gas chromatography. Most commercially available samples of CLA, prepared by base-catalyzed isomerization of linoleic acid (9*c*,12*c*-18:2), are composed of mixtures of four major isomers. While these isomers have been characterized, we found significant changes in CLA isomer ratios within samples obtained from the same producer/commercial supplier over a period of 1.5 yr. In the first sample, the four *cis/trans* isomers (8*t*,10*c*-18:2, 9*c*,11*t*-18:2, 10*t*,12*c*-18:2 and 11*c*,13*t*-18:2) were present in a ratio of approximately 1:2:2:1, while in the second sample they were present in almost equal proportions. If indeed certain daily levels of CLA intake are required to produce suggested health benefits in humans, changes in concentrations of specific CLA isomers could significantly impact these effects. Care must be taken to analyze the CLA used in human and animal studies.

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KEY WORDS: Conjugated, linoleic, CLA, composition, isomers, HPLC, GC

Conjugated linoleic acid (CLA; 9*c*,11*t*-18:2), present in dairy products and beef, and its isomers have been reported in animals to have anticarcinogenic, growth-promoting, antiatherogenic, antidiabetic, and lean body mass-enhancing properties (1–8). Most commercially available samples of CLA, prepared by base-catalyzed isomerization (9) of linoleic acid (9*c*,12*c*-18:2), are composed of mixtures of CLA isomers. While 9*c*,11*t*-18:2 is the primary CLA isomer found in nature (10), the "CLA" available in diet supplements is composed (11) primarily (>80% by weight) of four isomers (8*t*,10*c*-18:2, 9*c*,11*t*-18:2, 10*t*,12*c*-18:2, and 11*c*,13*t*-18:2).

Data from animal models have been used to suggest the 9*c*,11*t*-isomer is responsible for CLA's anticarcinogenic properties, whereas the 10*t*,12*c*-isomer is considered responsible for the observed weight loss/muscle-mass enhancement effects (12,13). The different CLA isomers present in commercially available diet supplements may thus have different health-related benefits, and other isomers (11*c*,13*t*-18:2, for example) may potentially be harmful (14). To determine the effects of specific CLA isomers, pure samples of each isomer are required. Yet CLA mixtures continue to be used (15).

We wish to report a second problem: significant batch-to-batch variations over time in CLA isomer ratios within samples obtained from the same source. Although researchers have noted significant variations in CLA composition (Ref. 16; from 0 to 75% CLA) in samples obtained from different suppliers, the existence of differences in composition of samples obtained from the same producer/commercial supplier has not been adequately addressed.

MATERIALS AND METHODS

Composition data from CLA samples obtained *ca.* 1.5 yr apart (June 1998 and December 1999) from the same commercial supplier were compared. The samples were obtained, in fatty acid form, in gel capsules. In each instance, 3 × 1 g capsules were opened, the contents combined, and one 30-mg sample was removed. The fatty acids were converted to fatty acid methyl esters with diazomethane (17) and analyzed in duplicate utilizing silver ion high-performance liquid chromatography (Ag-HPLC) [two Varian ChromSpher Lipids (C) columns (Varian Chrompack B.V., Middelburg, The Netherlands) linked in series; flow rate of 1.0 mL/min of 0.1% acetonitrile in hexane; ultraviolet detection at 233 nm] and gas–liquid chromatography [GC; Varian 3400; 100 m SP2380 (C) capillary column (Supelco, Inc., Bellefonte, PA); flame-ionization detector].

RESULTS AND DISCUSSION

The results are summarized in Table 1. Analysis by Ag-HPLC yielded much improved separation of the CLA isomers than we observed utilizing GC. With Ag-HPLC, the *cis/trans* isomers were separated into four peaks (six peaks in the year 1999 sample), the *trans/trans* isomers into six peaks, and the *cis/cis* isomers into four peaks, all resolved at >90%. When GC was utilized, the *trans/trans* isomers were resolved into only two peaks (the 11,13-/12,14-isomers; then the 7,9-, 8,10-, 9,11-, and 10,12-isomers), the *cis/trans* isomers into three peaks (9*c*,11*t*- and 8*t*,10*c*-18:2 eluted as one peak), and the *cis/trans* and *cis/cis* peak patterns tended to verlap. (Elution order was: *cis/trans*, *cis/cis*, then *trans/trans*.)

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Abbreviations: Ag-HPLC, silver ion high-performance liquid chromatography; CLA, conjugated linoleic acid; GC, gas–liquid chromatography.

TABLE 1
Conjugated Linoleic Acid (CLA) Compositions

	Sample-Analyzed 6/1998				Sample-Analyzed 1/2000			
	Gas chromatography		Ag-HPLC		Gas chromatography		Ag-HPLC	
CLA Isomer[a]	1[b]	2[b]	1[b]	2[b]	1[b]	2[b]	1[b]	2[b]
12t,14t-18:2	0.8[c]	0.7[c]	0.3	0.2	1.3[c]	1.3[c]	0.6	0.6
11t,13t-18:2			0.6	0.6			1.5	1.4
10t,12t-18:2	6.0[d]	6.1[d]	2.1	2.3	7.1[d]	7.1[d]	3.2	3.0
9t,11t-18:2			1.9	1.9			3.0	2.5
8t,10t-18:2			0.3	0.3			1.4	1.0
7t,9t-18:2			0.2	0.2			0.9	0.8
13c,15t-18:2?			0.0	0.0			0.6	0.8
12t,14c-18:2?			0.0	0.0			0.2	0.4
11c,13t-18:2	16.9	17.1	16.6	15.9	23.6	24.0	20.6	21.4
10t,12c-18:2	31.2	30.6	31.4	31.7	22.1	22.2	23.5	23.5
9c,11t-18:2	39.0[e]	38.1[e]	27.1	26.9	35.7[e]	36.4[e]	20.2	19.6
8t,10c-18:2			14.0	14.5			18.6	17.9
11c,13c-18:2	3.9	4.6	0.9	0.8	1.9	0.7	1.5	1.6
10c,12c-18:2			2.1	2.4	3.9	2.0	1.7	2.3
9c,11c-18:2	2.2	2.8	1.9	1.9	2.4	3.9	1.2	1.8
8c,10c-18:2			0.6	0.4	2.0	2.4	1.3	1.4
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Total:	*trans/trans*	*cis/trans*	*cis/cis*	TOTAL CLA[f]	Total:	*trans/trans*	*cis/trans*	*cis/cis*	TOTAL CLA[f]
GC	6.7	87.1	6.1	56.8	GC	8.4	81.4	10.1	62.2
	6.8	85.8	7.4	56.2		8.6	82.6	8.9	61.0
Ag-HPLC	5.4	89.1	5.4		Ag-HPLC	10.6	82.9	5.7	
	5.5	89.0	5.5			9.2	82.4	7.1	

[a]CLA isomers listed as eluted from silver-high-performance liquid chromatography (Ag-HPLC) as fatty acid methyl esters.
[b]Run number.
[c]Sum 11t,13t- and 12t,14t-18:2.
[d]Sum 7t,9t-, 8t,10t-, and 9t,11t-18:2.
[e]9c,11t- and 8t,10c-18:2 unresolved.
[f]Total CLA isomers (wt%) in sample. GC, gas–liquid chromatography.

Our "Total CLA" results (Table 1) determined by GC were in agreement with the "Total CLA" listed in the Material Safety Data Sheet (MSDS) included with the samples. We found the total percentage of conjugated isomers had increased *ca.* 10% by weight (GC data) from the year 1998 to the end of 1999 sample (16:0, 18:0, 9c-18:1, and 9c,12c-18:2 were also present at 35–40% by weight of total sample, with 9c-18:1 predominating at 23–25%). Between June 1998 and December 1999, the total percentage of *cis/cis* and *trans/trans* isomers increased slightly, and the % *cis/trans* totals decreased. The greatest change, however, was noted in the relative percentages of the four predominant *cis/trans* isomers (8t,10c-18:2, 9c,11t-18:2, 10t,12c-18:2, and 11c,13t-18:2). In the 1998 sample, the four *cis/trans* isomers were present in a ratio of approximately 1:2:2:1, but in the 1999 sample they were present in almost equal proportions. The presence of two other isomers (12t,14c- and 13c,15t-18:2?) in the 1999 sample is another indication that more "extreme" isomerization conditions were employed.

A quotation from a recent (18) paper, "CLA typically used in animal studies is prepared by alkali isomerization from pure linoleic acid, and contains more than 95% CLA, mainly *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers (85–90%) along with other minor isomers (*trans,trans* or *cis,cis*)" might thus not be applicable to CLA obtained from other producers/suppliers. And if, indeed, certain daily levels of CLA intake are required to produce the suggested health benefits in humans, changes in concentrations of specific CLA isomers could significantly influence these effects. Care must be taken to analyze the CLA used. "Total % CLA" should not be considered a sufficient label for these materials.

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APPENDIX C

Chapter 5 of the book Advances in *Conjugated Linoleic Acid Research, Volume 2*, J. Sebedio, W.W. Christie, and R. Adolf, Eds., AOCS Press, Champaign, IL, 2002

Advances in Conjugated Linoleic Acid Research, Volume 2

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Chapter 5

Commercial Synthesis of Conjugated Linoleate

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Introduction

Conjugated linoleic acid (CLA) has been available as a health food supplement in soft gelatine capsules since 1995 in the United States, and more recently in several European countries and Japan. CLA products designed for food and animal feed additives are expected to appear on the market in the near future. CLA has been produced for decades for technical purposes and continues to be used as a substitute for Chinese tung oil in the paint and varnish industry due to its "drying" characteristics. The production methods developed for technical CLA products were rapidly modified and improved upon after the discovery of the biological activity of the substance. This chapter will focus on supplements in particular, including current production methods, stability, and breakdown products. Purified isomers are currently available only for research purposes, but a few references to methods available for purification will be given.

CLA for Technical Applications

Dehydration of Ricinoleic Acid

Several decades ago, only two natural oils (tung oil and oiticica) were known to contain conjugated double bonds. Oils that contain these bonds rapidly form a polymer film ("drying") if a thin layer is exposed to air; tung oil was widely used in the paint and varnish industry. An increasing demand for such oils and limited availability encouraged efforts to produce drying oils from nonconjugated oils.

The main constituent of castor bean oil is ricinoleic acid (12-hydroxy-9-octadecenoic acid). Around 1937, dehydrated castor oil appeared on the market in the United States as a substitute for tung oil. Ten years later the product was established as one of the most popular drying oils (1). It has been known since 1888 that castor oil could be dehydrated, and since 1914 it was known that the main isomers of linoleic acid formed had double bonds at positions 9,11 and 9,12, but the detailed composition of dehydrated ricinoleic acid was not investigated until recently. A German patent from 1930 (2) and a U.S. patent from 1934 (3) describe the preparation of dehydrated castor bean oils. A modified procedure was recently used to produce an 83% pure 9-*cis*,11-*trans* CLA concentrate from purified ricinoleic acid (4). Main impurities were the 9-*cis*,11-*cis* and 9-*cis*,12-*trans*-octadecadienoic acids. Conventional dehydration

using high temperatures will create other isomers, mainly 8-*trans*,10-*cis* and *trans*, *trans* isomers. CLA from dehydrated castor oil is not available on the market in supplement form. Apart from safety issues, the reason is the absence of 10-*trans*,12-*cis* CLA, the isomer shown to inhibit fat synthesis (5).

Alkali Isomerization of Linoleic Acid Oils

Attempts to produce drying oil from nonconjugated oils were successful in the late 1930s as well as for oils containing methylene-interrupted fatty acids. In 1941, a U.S. patent was issued that describes the use of monohydric and polyhydric alcohols as solvents and a variety of alkaline catalysts (6). A few years later, two patents were issued that described the use of water (7) and steam (8), respectively, as solvent in an autoclave to achieve the temperatures necessary to conjugate unsaturated acids. It is actually the soap that is conjugated. Upon addition of mineral acid, the conjugated free fatty acids are liberated. Currently, CLA is produced for technical purposes in high alkaline water at ~230°C. Feedstock is usually free fatty acids (after fat splitting to recover glycerol). The product is usually distilled to yield a virtually colorless oil.

Production of CLA for Animal and Human Consumption

Alkaline Water Isomerization

The first products to appear on the health food market contained ~65% CLA, and the profile of the CLA isomers was similar to technical-grade products. Christie *et al.* (9), showed that the main isomers of CLA in addition to 9-*cis*,11-*trans* and 10-*trans*,12-*cis* were an 8,10 and an 11,13 isomer *cis*,*trans* or *trans*,*cis*. These were later identified as 8-*trans*,10-*cis* and 11-*cis*,13-*trans* (10). Such products are still available as supplements, and most if not all are produced from linoleate-rich starting materials in high-alkaline water reactions at temperatures >230°C. We investigated reaction parameters in water alkaline (KOH or NaOH catalyst) reactions trying to avoid formation of 11-*cis*,13-*trans* and 8-*trans*,10-*cis*. It turned out not to be possible to achieve a nearly quantitative isomerization and at the same time avoid formation of the said isomers (data not published).

Isomerization in Propylene Glycol

Quantitative isomerization of oils containing polyunsaturated fatty acids in monohydric and polyhydric alcohols was described in 1941 (6). A detailed procedure using ethylene glycol is described in a patent from 1996 (11). Ethylene glycol has not been used commercially for production of CLA for consumer safety reasons. Propylene glycol has therefore been selected by several producers who independently developed proprietary procedures (12,13). KOH was selected as catalyst because of its high solubility compared with NaOH. Reaction temperatures are typically 130–180°C, and times of reaction are from 3 to >24 h. Th

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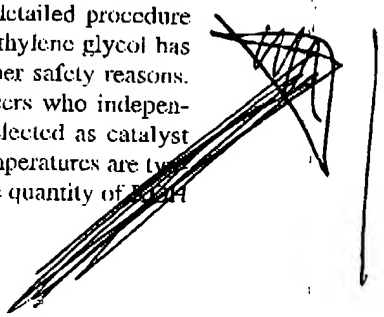

is substantial and in excess of that needed for quantitative saponification. After the reaction is complete, the mixture is cooled down and water and mineral acid (hydrochloric or sulfuric) are added. Free fatty acids of CLA are liberated as soon as the mixture becomes acidic. One patent describes the use of hexane at this point to extract CLA and facilitate separation from the bottom aqueous layer without emulsion problems. However, the operation is possible without the use of hexane. For the sake of recovery of propylene glycol, free fatty acids are preferred as feedstock oil. A triacylglycerol feedstock will create glycerol to contaminate the propylene glycol. After water and solvent (hexane if used) have been removed under vacuum, the CLA product is preferably purified by deodorization and distillation. Peroxides and volatiles are easily removed by deodorization. The peroxides are broken down to secondary volatile products that are removed in the process.

The purification process should also include a molecular distillation step to remove nonvolatile compounds such as polymers, sterols, and propylene glycol esters. Heavy metals could also arise from the isomerization process if mineral acids are used in stainless steel reactors (14). Their concentrations are reduced upon molecular distillation as well. A distilled product is almost colorless and has an acid value of ~200 (mg KOH/g). A nondistilled product might have an acid value of ~190, be yellow to slightly brown in color and have an opaque appearance. However, we have observed a slight decrease in acid value in capsules over time and also a darkening of the oil if the capsule material is colored. Due to the strong alkaline process, free fatty acids are the final product regardless of the form of feedstock (free fatty acid, a monoalkyl ester, or a triacylglycerol oil). Therefore, CLA in supplements are offered almost exclusively as free acids, in contrast to n-3 concentrates that are offered either as ethyl esters or reesterified triacylglycerols.

Isomerization of Mono-Alkyl Esters Using Alkali Metal Alcoholates

Recently, a proprietary method has been developed that quantitatively isomerizes methyl esters and ethyl esters of linoleic acid using very low quantities of catalysts and virtually no solvents (data not published). Because of the quantity of catalyst (~2%), only a small fraction of the ester is saponified and hence appears as free fatty acid after addition of a neutralizing agent. Most of the product (>92%) is still in the form of the methyl or ethyl ester after the isomerization process. The reaction proceeds at temperatures down to below 100°C, and the CLA product is characterized by very low levels of CLA isomers produced by thermal [1,5] sigmatropic rearrangements (see below).

Thermal [1,5] Sigmatropic Rearrangements of CLA Isomers

Production of CLA in propylene glycol or other alcohol under mild conditions gives rise to <0.5% each of the isomers 11-*cis*,13-*trans* and 8-*trans*,10-*cis*. After purification of single isomers, we showed that upon heating to 220°C in an inert atmosphere, 10-*trans*,12-*cis* gives rise to 11-*cis*,13-*trans* (Fig. 5.1). Upon heating an 11-*cis*,13-*trans* concentrate, 10-*trans*,12-*cis* was produced. Under optimal condi-

tions, an equilibrium is established between these isomers, and only minor quantities of *cis,cis* and *trans,trans* isomers are formed. The isomer shift is actually a thermal [1,5] sigmatropic rearrangement, (Fig. 5.2) allowed according to the orbital symmetry theory (Woodward-Hoffmann). For this sigmatropic rearrangement to occur, it is essential that one of the bonds be in the *cis*-configuration. A similar rearrangement is observed for the isomers 9-*cis*,11-*trans* and 8-*trans*,10-*cis* The phenomenon is actually a tool for chemists to produce new isomers. Any given CLA isomer that contains one double bond in the *cis*-configuration and one in the *trans*-configuration can be heated to be isomerized into another specific *cis,trans* or *trans,cis* isomer. Isomers formed might be predicted from formulae as in Fig. 5.2. A simple rule of thumb is that the two double bonds will move against the *cis* end of the bond pairs. For example, 7-*trans*,9-*cis* (a common isomer in milk fat) will isomerize to 8-*cis*,10-*trans* and *vice versa*. Prolonged heating of isomers

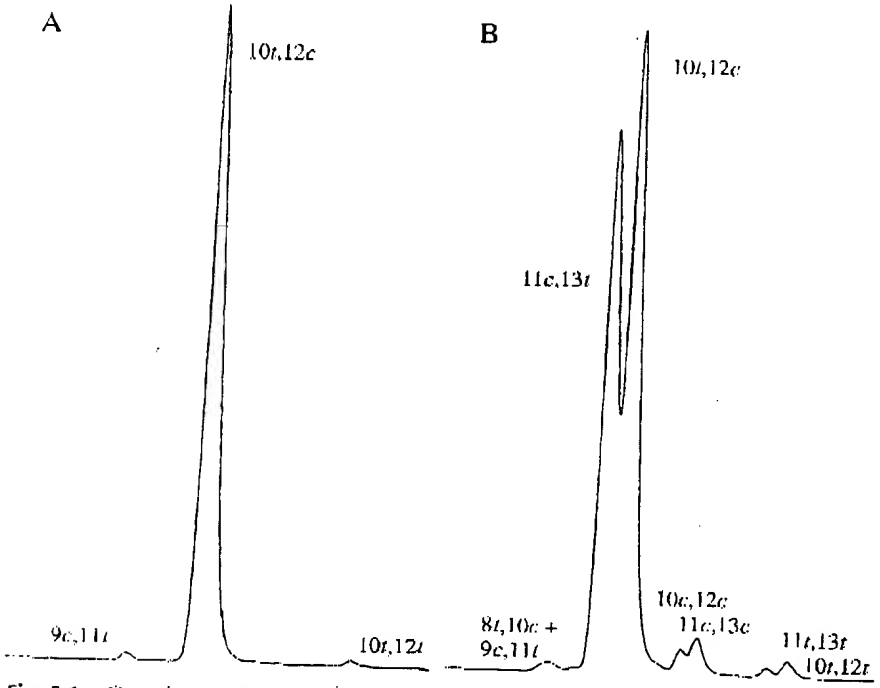

Fig. 5.1. Gas chromatography (GC) profile of ethyl ester of purified 10-*trans*,12-*cis* CLA isomer (a) before and (b) after heating to 220°C in an inert atmosphere for 2 h. The process caused isomerization into the isomer 11-*cis*,13-*trans* by thermal [1,5] sigmatropic hydrogen shift. GC conditions: 100-m CP Sil 88 fused silica capillary column and flame ionization detection (FID).

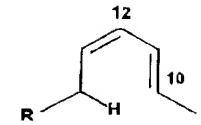

Fig. 5.2. Drawing explain
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Isomer Profile in Avail

The total content of CL
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exclusively 9-*cis*,11-*trans*
50% of the CLA. The for
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January-March 2002 by o
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Stability and Breakd

Stability of CLA Compa

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11-*trans* and 8-*trans*,10-*cis*
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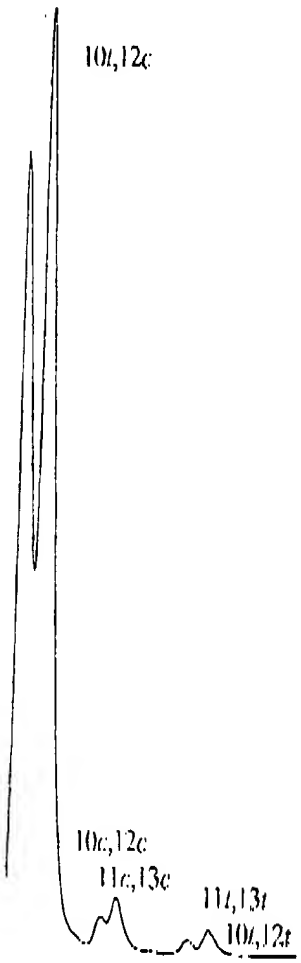


r of purified 10-*trans*,12-*cis*
an inert atmosphere for 2 h.
13-*trans* by thermal [1,5] sig-
38 fused silica capillary col-

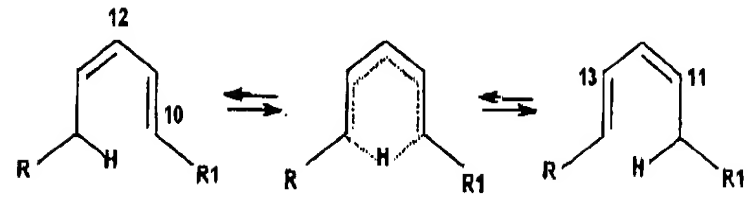


Fig. 5.2. Drawing explaining thermal [1,5] sigmatropic rearrangement between the CLA isomers 10-*trans*,12-*cis* and 11-*cis*,13-*trans*. Reaction is spontaneous and the transition state depicted in the middle is not an intermediate product. R= $-(CH_2)_4$ and R_1 = $-(CH_2)_8CO_2H$.

seems to gradually develop *cis,cis* and *trans,trans* isomers. Impurities present (iron, copper and other metals) will greatly favor formation of *trans,trans* isomers.

Isomer Profile in Available Supplements

The total content of CLA in supplements more or less reflects the starting material. Sunflower oil as a starting material results in ~65% CLA, whereas safflower oil yields up to 80%. Both oils contain a level of palmitic acid that tends to cause precipitation below room temperature. Products are now available with a reduced content of saturated acids and >80% CLA. The products can be classified in two groups, the "4-isomer product" and the "2-isomer product" (Fig. 5.3). The latter product contains almost exclusively 9-*cis*,11-*trans* and 10-*trans*,12-*cis*, both up to ~38% of the oil, or almost 50% of the CLA. The former, however, contains several isomers. The elution order on gas chromatography (GC) of the 4 main peaks is 9-*cis*,11-*trans*; 8-*trans*,10-*cis* (may co-elute with 9-*cis*,11-*trans*);11-*cis*,13-*trans*; and 10-*trans*,12-*cis* (9). In addition a major *trans,trans* peak (9,11 and 10,12 co-eluting) often reaches the same level. Such products may contain as little as 8% 10-*trans*,12-*cis*. Despite co-elution, the content of 8-*trans*,10-*cis* can be estimated approximately by measurement of 11-*cis*,13-*trans*. Both are produced to the same degree from their mother components. In other words, the ratio of 11-*cis*,13-*trans* to 11-*cis*,13-*trans* + 10-*trans*,12-*cis* equals that of 8-*trans*,10-*cis* to the co-eluting peak 8-*trans*,10-*cis* + 9-*cis*,11-*trans* (data not published). Products from a single source have been reported to show substantial variation in isomer profile (15), and products also are available that contains virtually no (present data, Table 5.1) or totally lack CLA (10). Two of 17 products sampled and analyzed in January-March 2002 by our laboratory contained high levels of the isomers 11-*cis*,13-*trans* and 8-*trans*,10-*cis* (Table 5.1).

Stability and Breakdown Products f CLA Preparations

Stability of CLA Compared with Linoleic Acid

A few studies report data on the stability of CLA compared with linoleic acid in different test models. Bubbling of oxygen through samples at 90°C resulted in a

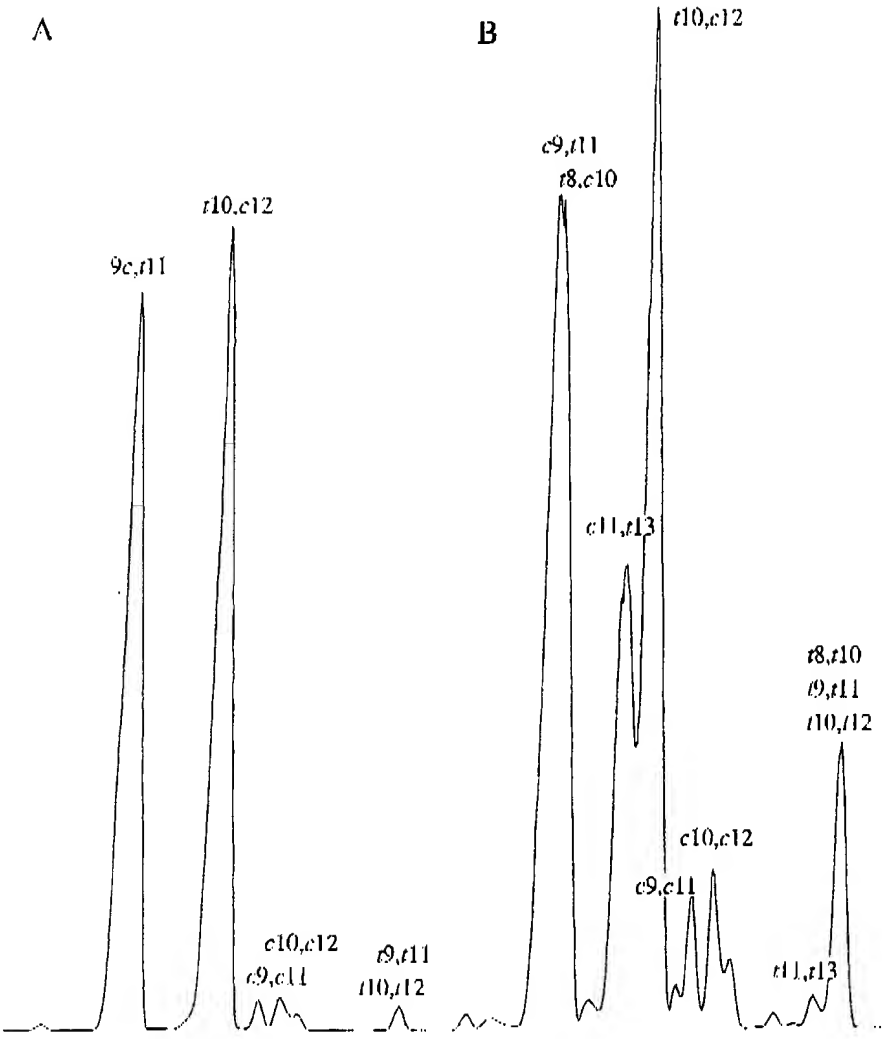


Fig. 5.3. Partial gas chromatography (GC) profile of ethyl esters of (a) a "2 isomer type" and (b) a "4 isomer type" CLA supplement, using a 100-m CP Sil 88 fused silica capillary column and flame ionization detection (FID). Product (a) is identical to product No. 14 and product (b) is identical to No.17 in Table 5.1. Note co-elution of *8-trans,*10-*cis* and 9-*cis,*11-*trans*.

much higher peroxide value (PV) in linoleic acid (16) than for CLA. When a mixture of CLA isomers was heated to 50°C in air, the rate of oxidation was considerably faster for CLA than for linoleic acid. The rate of oxidation was measured

TABLE 5.1
Content of CLA (% of Tot
January–March 2002[a]

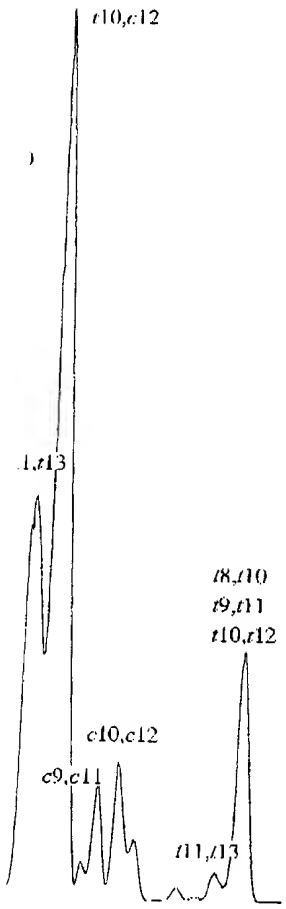
Product	Product type
1	Soft gelatine cap
2	Liquid
3	Soft gelatine cap
4	Soft gelatine cap
5	Soft gelatine cap
6	Soft gelatine cap
7	Soft gelatine cap
8	Soft gelatine cap
9	Soft gelatine cap
10	Soft gelatine cap
11	Soft gelatine cap
12	Soft gelatine cap
13	Soft gelatine cap
14	Soft gelatine cap
15	Liquid, emulsion
16	Soft gelatine cap
17	Soft gelatine cap

[a]The isomers 10-*trans*,12-*cis* and
ucts were of the "4 isomer" type.
9-*cis*,11-*trans* (not tabulated due
trans,12-*cis* in all supplements co
mg KOH/g. (A 100.00% free fatty
region of product 14 and produc

CLA was more stable tha
esters (18). Another stud
the following order: olea
40°C and monitored by
cis,11-*trans*, the major m
and 13-monohydroperox
13-, and 14-monohydrope

Data reported on the
tions. CLA do not easily
seems comparable to that
breakdown of peroxides i

Volatiles



yl esters of (a) a "2 isomer
100-m CP Sil 88 fused silica
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s > *cis,cis*. (17). In a study
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TABLE 5.1
Content of CLA (% of Total) in 17 Commercial Supplements Sampled in January–March 2002[a]

Product	Product type	Country	%CLA	%10*t*,12*c*	%11*c*,13*t*	Acid value
1	Soft gelatine capsule	Norway	80.1	47.8	0.4	197
2	Liquid	Norway	78.6	47.1	1.8	2
3	Soft gelatine capsule	Norway	69.1	46.7	1.2	196
4	Soft gelatine capsule	Norway	78.3	48.7	0.3	197
5	Soft gelatine capsule	Norway	76.4	46.6	1.3	193
6	Soft gelatine capsule	U.S.	71.4	46.3	0.5	189
7	Soft gelatine capsule	U.S.	74.8	43.1	0.9	192
8	Soft gelatine capsule	U.S.	77.9	48.5	0.3	199
9	Soft gelatine capsule	U.S.	70.8	44.4	0.6	189
10	Soft gelatine capsule	U.S.	79.6	45.3	0.4	193
11	Soft gelatine capsule	U.S.	77.0	44.4	2.3	192
12	Soft gelatine capsule	U.S.	74.3	43.6	1.0	187
13	Soft gelatine capsule	U.S.	61.5	28.5	0.8	180
14	Soft gelatine capsule	U.S.	76.3	48.4	0.3	196
15	Liquid, emulsion	U.S.	1.2	47.8	0.3	NA
16	Soft gelatine capsule	S. Africa	51.7	16.5	16.7	198
17	Soft gelatine capsule	Norway	57.7	29.9	16.5	200

[a]The isomers 10-*trans*,12-*cis* and 11-*cis*,13-*trans* are expressed as the percentage of total CLA. Only two products were of the "4 isomer" type. Two products were liquids, one oil and one emulsion (1.7% fat). Content of 9-*cis*,11-*trans* (not tabulated due to overlap with 8-*trans*,10-*cis*) is approximately equal or slightly less than 10-*trans*,12-*cis* in all supplements currently available. Distilled products typically have acid values of 195–200 mg KOH/g. (A 100.00% free fatty acid product of oleic acid has a theoretical acid value of 198.60). CLA region of product 14 and product 17 is illustrated in Figure 5.3. NA, not available.

CLA was more stable than linoleic acid as free fatty acids, and less stable as ethyl esters (18). Another study using methyl esters reported that stability decreased in the following order: oleate > CLA > linoleate. Samples were stored in the dark at 40°C and monitored by thin-layer chromatography (TLC), GC and PV. From 9-*cis*,11-*trans*, the major monohydroperoxides formed were identified as 8-, 9-, 12- and 13-monohydroperoxides, whereas 10-*trans*,12-*cis* yielded primarily 9-, 10-, 13-, and 14-monohydroperoxides (19).

Data reported on the PV of CLA preparations are consistent with our observations. CLA do not easily develop high PV, yet the oxidative breakdown of CLA seems comparable to that of linoleic acid. The reason is likely to be a more rapid breakdown of peroxides into secondary oxidation products.

Volatiles

In a pilot project on developing a procedure for CLA production, a high content of hexane was observed in a product by headspace GC-mass spectrometry. After searching for the source of contamination, it was finally concluded that pentane

and hexane are among the secondary oxidation products of CLA. This was later confirmed by experiments. To our knowledge, hexane has never been reported to be an important inherent oxidation product of vegetable oils. In a free fatty acid concentrate of 9-*cis*,11-*trans* stored in the dark with air access for 1 wk, the two major volatiles that developed were, not surprisingly, heptanal and 2-nonenal. The concentration increased from 4.8 and 0.7 to 84.6 and 22.5 µg/g, respectively. Volatile breakdown products seem not to build up in soft gelatine capsule supplements. A CLA product that was stored for 5 y at room temperature contained 2.3 µg/g hexanal and 2.2 µg/g heptanal (data not published). No antioxidant was added to the supplement.

Among less volatile breakdown products, furan fatty acids were reported when air was bubbled through CLA dissolved in a mixture of methanol and water at 50°C. (20). Furanoid fatty acids might also arise in preparation of fatty acid methyl esters (FAME) for GC. To our knowledge, furan fatty acids have not been reported as an oxidative breakdown product in dry oil preparations of CLA.

Polymers

Conjugated oils are considered valuable raw materials for the paint and varnish industry because of their film forming properties ("drying") upon air access. This property gives rise to concern regarding the stability of CLA preparations. In a stability test program, 10 mL of CLA triacylglycerols and free fatty acids were stored in an amber open glass bottle in darkness. After 4 mo at 25°C, controls without antioxidants added were highly viscous and not suitable for further stability testing. The samples had a membrane layer on the surface, and the viscosity clearly developed over time. Samples with antioxidants did show a retarded viscosity development (data not published).

Soft gelatine capsules are considered to give reasonable protection from exposure of unsaturated oils to air. Capsules containing CLA free fatty acids showed a slight increase in polymer content from 1% in freshly prepared capsules to 7% after 5 y (data not published). For comparison of health risks, a limit for rejection on cooking oils has been established in some countries; values listed in a report from the European Parliament are 16% (Holland), and 10% (Belgium and Czech Republic) (21).

Stability of CLA in Soft Gelatine Capsules

No data have yet been published on the stability of CLA in capsules. Observations on polymers and volatiles in capsules are reported above. In a stability test program according to International Conference on Harmonization (ICH) guidelines on a free fatty acid product, the content of total CLA was not significantly reduced after 24 mo at 25°C/60% relative humidity. In this test, CLA was measured by GC. Peroxide value (PV) did not develop in the capsules (data not published).

Next Generation Pr

Isomer Purification

All CLA supplements cu
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Triacylglycerols for Foo

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Summary

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CLA was measured by GC.
a not published).

Next Generation Products

Isomer Purification

All CLA supplements currently offered contain approximately equal amounts of 9-*cis*,11-*trans* and 10-*trans*,12-*cis*. The extra costs of producing a biased isomer product might be justified if beneficial health effects were documented. The 9-*cis*,11-*trans* and the 10-*trans*,12-*cis* isomers of CLA are now available for research purposes in kilogram scale with a purity of ~90%. In small quantities, purities up to 99% are offered. High yields and high purity can be obtained by repeated crystallization of the methyl ester forms in acetone at temperatures as low as –60°C (22).

A concentrate with 83% 9-*cis*,11-*trans* isomer was obtained from gentle dehydration of ricinoleic acid from castor bean oil and subsequent purification steps (4). The use of urea inclusion compounds does not seem to be a feasible procedure to separate 9-*cis*,11-*trans* and 10-*trans*,12-*cis* (23). Enzymes, however, are promising tools for these separations. A 98% concentrate of 9-*cis*,11-*trans* was reported by using lipase from *Geotrichum candidum*. The enzyme was capable of esterifying selectively 9-*cis*,11-*trans* to monohydric alcohols from a mixture of several isomers (24). A patent has been issued on purification and characterization of isomerases from *Propionibacterium acnes* and *Clostridium sporogenes*. The purified isomerase preparations were able to quantitatively isomerize linoleic acid into the 10-*trans*,12-*cis* isomer of CLA (25).

Triacylglycerols for Food Applications

Free fatty acids and monoalkyl esters are applicable to supplement capsules and probably also to animal feed formulations. However, as an ingredient in food for human consumption, CLA is most attractive as a triacylglycerol. A nonspecific lipase has been reported to esterify CLA with glycerol very efficiently (26). Incorporation of CLA into food fats and oils has also been reported for fish oils (27), butterfat (28,29), and corn oil (30). A bottled triacylglycerol product, stabilized with antioxidants, has been available in the health food market in Scandinavia since 2000. Flavor and antioxidants are added to the oil designed to be taken by spoon. Further technical developments of CLA products improving the stability and applicability as well as addressing specific issues of food legislation will require attention before CLA can be made available as an ingredient for animal feed and human food.

Summary

CLA supplements for human consumption have been available since 1995, and most of the products contain between 60 and 80% CLA in the form of free fatty acids. The history of CLA produced for technical purposes dates back almost 100

y, however. The isomer profile of the supplements range from an almost pure 9-*cis*,11-*trans* + 10-*trans*,12-*cis*-50/50 mixture (made in alcohol solvents between 100 and 150°C), to a mixture with four prominent *cis,trans* or *trans,cis* isomers produced in high alkaline water at high temperatures, of which 8-*trans*,10-*cis* and 11-*cis*,13-*trans* 18:2 are produced from 9-*cis*,11-*trans* and 10-*trans*,12-*cis*, respectively, by thermal [1,5] sigmatropic rearrangements of the isomers. Supplements are typically offered as free fatty acids in soft gelatine capsules. Unpublished data on stability of CLA in capsules stored according to ICH guidelines for 2 y did not show any loss of active ingredient.

Acknowledgments

Per Christian Sæbø and his staff at the laboratory of Natural ASA is acknowledged for patient experimental work on CLA production and purification process developments for the last 5 years. Thanks to Prof. emeritus Lars Skattebøl for valuable comments on migration of sigma bonds.

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APPENDIX D

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Conjugated Linoleic Acid Modulates Tissue Levels of Chemical Mediators and Immunoglobulins in Rats

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ABSTRACT: The effects of conjugated linoleic acid (CLA) on the levels of chemical mediators in peritoneal exudate cells, spleen and lung, and the concentration of immunoglobulins in mesenteric lymph node and splenic lymphocytes and in serum were examined in rats. After feeding diets containing either 0 (control), 0.5 or 1.0% CLA for 3 wk, there was a trend toward a reduction in the release of leukotriene B_4 (LTB_4) from the exudate cells in response to the dietary CLA levels. However, CLA did not appear to affect the release of histamine. A similar dose-response pattern also was observed in splenic LTB_4, lung LTC_4 and serum prostaglandin E_2 levels, and the differences in these indices between the control and 1.0% CLA groups were all statistically significant. The reduction by CLA of the proportions of n-6 polyunsaturated fatty acids in peritoneal exudate cells and splenic lymphocyte total lipids seems to be responsible at least in part for the reduced eicosanoid levels. Splenic levels of immunoglobulin A (IgA), IgG, and IgM increased while those of IgE decreased significantly in animals fed the 1.0% CLA diet. This was reflected in the serum levels of immunoglobulins. The levels of IgA, IgG, and IgM in mesenteric lymph node lymphocytes increased in a dose-dependent manner, while IgE was reduced in those fed the higher CLA intake. However, no differences were seen in the proportion of T-lymphocyte subsets of mesenteric lymph node. These results support the view that CLA mitigates the food-induced allergic reaction.
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Conjugated linoleic acid (conjugated derivatives of linoleic acid, CLA) exerts diverse physiological effects most of which are favorable to human health. A range of studies has shown a marked alleviating effect of CLA on mammary carcinogenesis (1–4). The mechanism underlying this effect is not yet well understood (5), but continued intake of CLA is not necessarily required for suppression of carcinogenesis (6,7). When considering the diverse effects of CLA, it is reasonable that eicosanoids are involved in the mechanism. The influence of CLA on the metabolic processes leading from linoleic acid to arachidonic acid and, hence, eicosanoids appears to be related to their desirable effects, since CLA tended to reduce the tissue level of prostaglandin E_2 (PGE_2), a putative candidate for a cancer-promoting effect of dietary n-6 polyunsaturated fatty acids (PUFA) (8). In addition, there is a possibility that CLA itself serves as substrate of enzymatic systems for eicosanoid production, as it is shown to undergo desaturation and elongation similar to linoleic acid (9), although it is unknown whether these metabolites could be converted to eicosanoids.

Since the food allergic reaction can readily be modified by the type of dietary PUFA, either n-6 or n-3 (10,11), it is interesting to know if CLA could modify it. The clinical symptom of food allergy is induced by the production of chemical mediators such as histamine and leukotriene (LT) and PG triggered by allergen-specific immunoglobulin (Ig)E (12,13). Our previous studies showed a reduction by CLA of the serum PGE_2 level (8), which is one of the typical chemical mediators in the allergic reaction (12,13). In this context, Belury and Kempa-Steczko (14) showed that CLA reduces the proportion of linoleic acid dose-dependently in hepatic phospholipid and suggested this may result in modified arachidonate-derived eicosanoid production by extrahepatic tissues. More recently, Wong *et al.* (15) reported that CLA modulates certain aspects of the immune defense such as lymphocyte proliferation in mice, although the effect was not always reproduced possibly because of the dependence on the duration of the feeding period. In the present study, we measured the production of chemical mediators and the level of Ig in rats fed different levels of CLA, either 0.5 or 1.0%.

MATERIALS AND METHODS

Preparation of CLA. CLA was prepared according to the method described by Ip *et al.* (16). In brief, 50 g of linoleic acid, purity >99% (Sigma Chemical Co., St. Louis, MO) was dissolved in 290 g of ethylene glycol containing 15 g of NaOH and heated at 180°C for 2 h under nitrogen. After cooling to room temperature, the content was adjusted to pH 4 and extracted with *n*-hexane. The hexane layer was washed with 5% NaCl, dehydrated with 3-A molecular sieves (Nacalai

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Abbreviations: CLA, conjugated linoleic acid; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; LT, leukotriene; MLN, mesenteric lymph node; PEC, peritoneal exudate cells; PG, prostaglandin; POD, peroxidase; PUFA, polyunsaturated fatty acid.

Tesqu, Kyoto, Japan) and dried in a rotary evaporator under nitrogen. The purity of CLA was measured by gas–liquid chromatography (Shimadzu GC-17A, Kyoto, Japan) using a Supelcowax 10 column (0.32 mm × 60 m, film thickness, 0.25 μm; Supelco Inc., Bellefonte, PA). Column temperature was raised from 150 to 220°C at a rate of 4°C/min. The identification of peaks was carried out by the equivalent chain length method (17) and gas chromatography–mass spectrometry (Jeol Auto MS 50, Tokyo, Japan). The purity of CLA preparation was 80.7% with the following composition in percentage: 9*c*,11*t*/9*t*,11*c*, 29.8; 10*t*,12*c*, 29.6; 9*c*,11*c*, 1.3; 10*c*,12*c*, 1.4; 9*t*,11*t*/10*t*,12*t*, 18.6; linoleic acid, 5.6; and others, 13.7.

Animals and diets. The animal experiment adhered to the Kyushu University guidelines for the care and use of laboratory animals. Male, 4-wk-old Sprague-Dawley rats were obtained from Japan Charles River (Atsugi, Japan) and housed individually in a room with controlled temperature and light (20–23°C and lights on 0800–2000 h). After acclimation for 4 d, rats were divided into three groups of 10 rats which were given free access to the experimental diets. The diets were prepared according to the recommendation of the American Institute of Nutrition (AIN-93G diet) (18). The basal diet contained the following ingredients, in g/100 g diet: cornstarch 39.8; casein, 20.0; dextrinized cornstarch, 13.2; sucrose, 10.0; soybean oil, 7.0; AIN-93 mineral mixture, 3.5; AIN-93 vitamin mixture, 1.0; L-cystine, 0.3; choline bitartrate, 0.25; cellulose, 5.0; *tert*-butylhydroquinone, 0.002; and either linoleic acid, 1.0; linoleic acid (Control) and CLA, 0.5 and 0.5; or CLA, 1.0. Thus, LA or CLA was added at the expense of soybean oil in the AIN-93G diet. The fatty acid composition calculated from the composition of individual oils is given in Table 1. Body weight and food intake were recorded every other day. After 3 wk of feeding, five rats were used for collection of the exudate cells and the remaining five rats for other analyses. Blood was withdrawn from the abdominal aorta under light diethyl ether anesthesia and tissues were immediately excised.

Preparation of peritoneal exudate cells (PEC). The method described by Matsuo *et al.* (19) was adopted for the preparation of PEC. Tyrode buffer, consisting of 137 mM NaCl, 2.7 mM KCl, 0.4 mM $NaH_2PO_4 \cdot 2H_2O$, 1 mM $MgCl_2 \cdot 6H_2O$, 12 mM $NaHCO_3$, 1.8 mM $CaCl_2 \cdot 2H_2O$, 5.6 mM D-glucose and 0.1% bovine serum fraction V (Boehringer Mannheim GmbH, Mannheim, Germany), pH 7.4, was injected into the rat peritoneal cavity (6 mL/100 g body weight), and the abdomen was gently massaged for 2 min. Then, the cavity was opened, and the buffer containing PEC was recovered with a plastic pipet. The fluid was centrifuged at 200 × *g* for 5 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in Tyrode buffer.

Measurement of leukotriene B_4 (LTB_4) and histamine. LTB_4 was measured as described elsewhere (20–22). PEC (2×10^6 cells) were suspended in Tyrode buffer containing 5 mM calcium ionophore A23187 (Sigma Chemical Co.). After incubating for 20 min at 37°C, 50 mL of the acetonitrile/methanol mixture (6:5, vol/vol) and 50 ng of PGB_2 (Sigma Chemical Co.), as the internal standard, were added. The mixture was kept at –20°C for 30 min and then centrifuged at 1,000 × *g* for 10 min. The supernatant was filtered through a 4-GV 0.22 μm filter (Millipore Corp., Tokyo, Japan). LTB_4 was measured by reversed-phase high-performance liquid chromatography (HPLC) (SCL-10A; Shimadzu Co., Kyoto, Japan) equipped with an ODS-A column (150 × 6.0 mm, 5 μL particle size; YMC, Kyoto, Japan). A mixture of acetonitrile/methanol/water (30:25:45, by vol) containing 5 mM CH_3COONH_4 and 1 mM disodium EDTA, pH 5.6, was used as a mobile phase with a flow rate of 1.1 mL/min. LTB_4 and PGB_2 were detected by absorbance at 280 nm (SPD-10A; Shimadzu Co.). Quantitation of LTB_4 was achieved by comparing the peak area of LTB_4 with that of PGB_2. Histamine content in the culture supernatant was measured fluorometrically (19,23). The intracellular content of histamine also was measured after disrupting the cells by sonication.

Preparation of spleen and mesenteric lymph node (MLN) lymphocytes. Spleen and MLN were excised immediately after withdrawing blood from the aorta, and the tissues were immersed in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) (24,25). The lymphocytes were rinsed three times with the RPMI 1640 medium and filtered to remove tissue scum. To remove fibroblasts, cell suspension was incubated for 30 min at 37°C. Then, 5 mL of the resulting cell suspension was layered on 4 mL of Lympholyte-Rat (Cedarlane, Hornby, Canada) and centrifuged at 1,500 × *g* for 30 min. The lymphocyte band at the interface was recovered, and the cells were rinsed again. The lymphocytes were cultured in 10% fetal bovine serum (Intergen, Purchase, NY) in RPMI 1640 medium at a cell density of 2.5×10^6 cells/mL with or without 2.5 μg/mL of lipopolysaccharide (Bacto lipopolysaccharide B, *Escherichia coli* 026:B6; Difco Laboratories, Detroit, MI). After incubation at 37°C for 24 and 72 h, the concentrations f IgA, IgG, IgM, and IgE were measured by an enzyme-linked immunosorbent assay (ELISA) (26).

T-cell population analysis. Spleen and MLN lymphocytes were analyzed as $CD4^+$- and $CD8^+$-cells by using fluorescein-labeled mouse anti-CD4 (W3/25, mouse IgG1) or phycoerythrin-labeled mouse anti-CD8 (MRC OX-8, mouse IgG1) (both from Serotec Ltd., Kidlington, Oxford, United King-

TABLE 1
Fatty Acid Composition of Dietary Fat[a]

Fatty acid	Group		
	Control	0.5% CLA	1.0% CLA
16:0	9.1	9.0	9.0
18:0	3.2	3.42	3.2
18:1	20.4	20.3	20.1
CLA	—	6.4	12.9
18:2	59.7	53.6	47.4
18:3	7.5	7.5	7.4

[a]Fatty acid composition was calculated from the composition of individual component fats, soybean oil, linoleic acid, and conjugated linoleic acid (CLA).

dom) (23,25). The stained lymphocytes were fixed with 2% paraformaldehyde and analyzed with the EPICS Profile II flowcytometer (Coulter Electronics Ltd., Luto, United Kingdom).

Measurement of serum and culture supernatant Ig by ELISA. Measurements of total Ig were executed using sandwich ELISA methods (24,25). Goat anti-rat IgE, rabbit anti-rat IgG (Fab')2, goat anti-rat IgM (all from Biosoft, Paris, France), and mouse anti-rat IgA (Zymed Lab, San Francisco, CA) were used to fix respective Ig. These antibodies were diluted 1,000 times with 50 mM carbonate-bicarbonate buffer (pH 9.6), and each well of 96-well plates was treated with 100 µL of the solution for 1 h (2 h for IgA) at 37°C. After blocking with 300 µL of the blocking solution overnight at 4°C, each well was treated with 100 µL of the diluted serum or culture supernatant for 1 h (2 h for IgA) at 37°C. Bound IgA was detected by reacting stepwise with 100 µL of peroxidase (POD)-conjugated rabbit anti-rat IgA (1,000 times dilution; Zymed) at 37°C for 2 h, IgG with 100 µL of POD-conjugated rabbit anti-rat IgG (Fab')2 (2,000 times dilution; Cappel, West Chester, PA), and IgM with 100 µL of POD-conjugated goat anti-rat IgM (1,000 times dilution, Cappel) at 37°C for 1 h. Wells were rinsed three times with Tween-20 in phosphate-buffered saline between each step. After incubation at 37°C for 15 min with 100 µL of 1.5% oxalic acid, absorbance at 415 nm was measured with an MPR-A4i ELISA reader (Tosoh, Tokyo, Japan). The bound IgE was detected by reacting with biotin-conjugated mouse anti-rat IgE (2,000 times dilution; Betyl, Montgomery, TX) followed by POD-conjugated avidin (5,000 times dilution, Zymed Lab) at 37°C for 1 h.

Statistical analysis. Data were analyzed by one-way analysis of variance followed by Duncan's new multiple-range test to identify significant differences (27). Values in the text are means ± SE.

RESULTS

Growth performance and tissue weight. As shown in Table 2, there was no difference in food intake and growth of rats for 3 wk among the groups. Thus, the feed efficiency also was comparable among the groups (mean values 0.41 to 0.42). Among tissues weighed, there was a tendency of increasing liver weight and decreasing perirenal adipose tissue weight by dietary CLA and the difference between the linoleic acid and 1.0% CLA groups was significant.

Release of chemical mediators from PEC. PEC isolated from rats fed linoleic acid or CLA were incubated with or without calcium ionophore A23187, and the concentrations of histamine and LTB_4 were measured in the medium. The content of histamine in the cells also was measured to estimate the cellular histamine contents. As shown in Figure 1, the effect of CLA on the release of histamine in PEC was diverse, and there was no significant difference in any of the parameters measured. However, the amounts of histamine stored in the cells tended to decrease with an increasing dietary level of CLA. There was a trend toward a reduction in

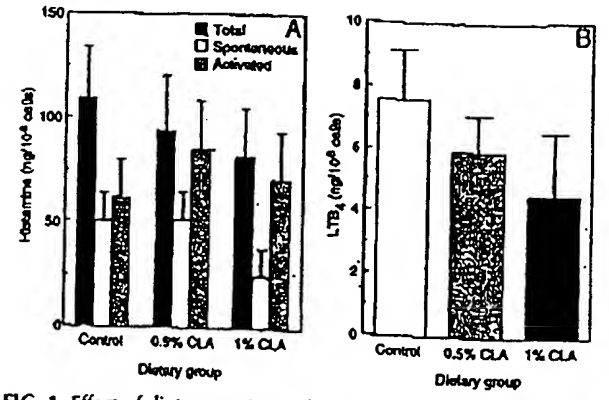

FIG. 1. Effect of dietary conjugated linoleic acid (CLA) on histamine content and release (A) and leukotriene B_4 (LTB_4) release (B) in rat peritoneal exudate cells. Means ± SE of five rats. Histamine release was measured in the presence and absence of calcium ionophore A23187. Total, total amounts of histamine in the cells; Spontaneous, the amount of histamine released during incubation without calcium ionophore A23187; Activated, the amount of histamine which was released from the cells when treated with A23187.

LTB_4 release in response to the dietary level of CLA, but the difference was not significant.

Tissue eicosanoid levels. The effect of CLA on LTB_4 and LTC_4 levels of spleen and lung is shown in Figures 2 and 3, respectively. CLA dose-dependently reduced the level of splenic LTB_4, and the difference between the control and 1% CLA groups was significant. No effect of CLA on the splenic LTC_4 level was observed. However, the concentration of LTC_4 in lung was reduced significantly by CLA even at the 0.5% dietary level. A trend of the dose-dependent reduction of LTB_4 also was observed, but the difference was not significant. The results of the levels of spleen and serum PGE_2 are summarized in Figure 4. CLA significantly reduced the concentration of serum PGE_2, while there was no effect of CLA on the splenic level of PGE_2.

Fatty acid compositions of PEC and splenic lymphocyte lipids. The PUFA composition of PEC and spleen lympho-

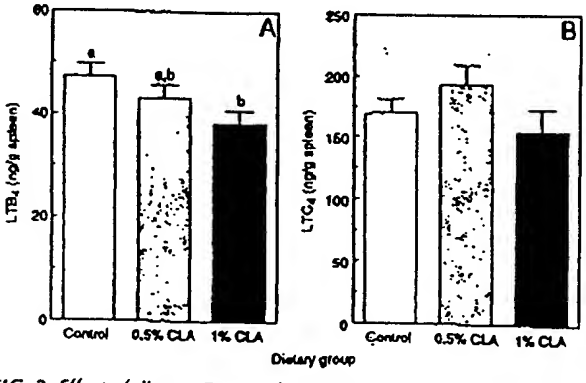

FIG. 2. Effect of dietary CLA on the concentration of splenic (A) LTB_4 and (B) leukotriene C_4 (LTC_4). Mean ± SE of five rats. Values without a common letter are significantly different at $P < 0.05$. For abbreviations see Figure 1.

TABLE 2
Effects of CLA on Growth and Tissue Weights of Rats[a]

Parameter	Group		
	Control	0.5% CLA	1.0% CLA
Initial body weight (g)	102 ± 1	101 ± 1	102 ± 1
Final body weight (g)	170 ± 2	166 ± 3	162 ± 4
Food intake (g/day)	19.1 ± 0.2	18.9 ± 0.3	18.6 ± 0.3
Tissue weight (g/100 g body weight)			
Liver	4.17 ± 0.09[a]	4.11 ± 0.09[a,b]	4.54 ± 0.07[b]
Kidney	0.85 ± 0.03	0.86 ± 0.03	0.87 ± 0.05
Perirenal adipose tissue	1.41 ± 0.07[a]	1.09 ± 0.09[a,b]	0.97 ± 0.14[b]
Heart	0.40 ± 0.02	0.34 ± 0.04	0.34 ± 0.04
Lung	0.48 ± 0.02	0.52 ± 0.02	0.49 ± 0.01
Spleen	0.22 ± 0.01	0.22 ± 0.01	0.25 ± 0.02
Brain	0.66 ± 0.02	0.70 ± 0.01	0.70 ± 0.01
Testis	0.96 ± 0.04	0.87 ± 0.10	1.00 ± 0.03

[a]Mean ± SE of 5 rats. Control group received 1.0% linoleic acid; 0.5% CLA group, 0.5% each of linoleic and CLA; and 1.0% CLA group, 1.0% CLA, respectively. Values without a common superscript letter (a,b) are significantly different at $P < 0.05$. For abbreviation see Table 1.

cyte total lipids is shown in Table 3. There was a dose-dependent reduction by dietary CLA of all n-6 PUFA, 18:2, 20:3, 20:4 and 22:4 in PEC lipids, while there was no difference in the proportion of n-3 PUFA, 22:6 among the groups. A clearer change in these n-6 PUFA was shown in splenic lymphocyte total lipids, and the reduction of 20:4n-6 was significant on a 1.0% CLA diet. Docosahexaenoic acid also tended to decrease with dietary CLA. The decreasing trend of all PUFA in CLA-fed rats was mainly attributable to a moderate increase in major saturated fatty acids, and oleic acid tended to decrease similar to PUFA (data not shown).

Serum thiobarbituric acid value. The concentration of thiobarbituric acid-reactive substance in serum was not modified by dietary CLA, and the values were within 4.1 to 5.5 ng/mL serum in all groups of rats.

Serum Ig levels. As shown in Figure 5, CLA increased the concentration of IgA, IgG and IgM, while decreasing that of IgE in serum. The difference between the control and 1.0% CLA groups was significant in these Ig.

Ig levels in spleen and MLN lymphocytes. Table 4 shows the Ig levels in the medium of rat spleen and MLN lymphocytes cultured for 72 h with or without lipopolysaccharide. Irrespective of the presence or absence of lipopolysaccharide, CLA showed no detectable effects on the Ig levels in spleen lymphocytes except for those of IgM after incubation with lipopolysaccharide, where CLA increased it in a dose-dependent manner. Under the similar situation, CLA increased the concentration of IgA, IgG, and IgM in MLN lymphocytes. The magnitude of the increase was particularly marked at the dietary CLA level of 1.0%. In contrast, there was a significant reduction of the IgE level when the cells from rats fed a 1% CLA diet were incubated with lipopolysaccharide in comparison with the control. A similar response to CLA also was observed even when these cells were incubated for 24 h (data not shown).

Subsets of MLN lymphocytes. The proportion of T-lymphocyte populations of MLN was analyzed as $CD4^+$ and $CD8^+$ subsets. There were no effects of CLA on their relative proportions ($CD4^+/CD8^+$ ratio; 2.6 ± 0.3, 2.4 ± 0.2, and 2.8 ± 0.1 for the control, 0.5% CLA, and 1.0% CLA, respectively).

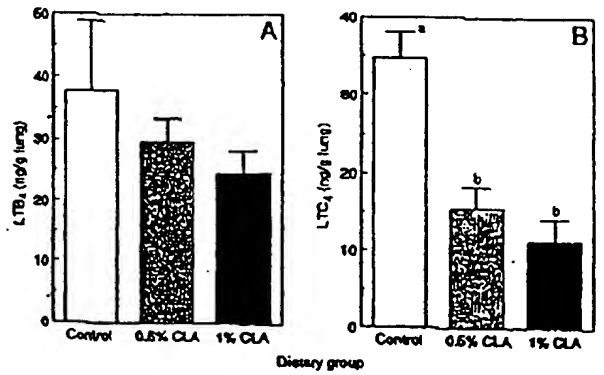

FIG. 3. Effect of dietary CLA on the concentration of lung (A) LTB_4 and (B) LTC_4. Mean ± SE of five rats. Values without a common letter are significantly different at $P < 0.05$. For abbreviations see Figures 1 and 2.

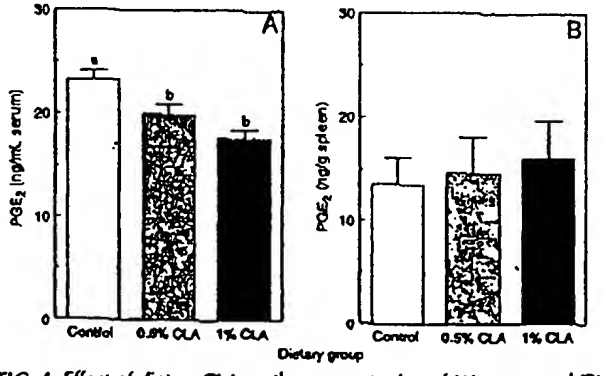

FIG. 4. Effect of dietary CLA on the concentration of (A) serum and (B) spleen prostaglandin E_2 (PGE_2). Mean ± SE of five rats. Values without a common letter are significantly different at $P < 0.05$. For other abbreviation see Figure 1.

TABLE 3
Effects of CLA on Polyunsaturated Fatty Acid Compositions of Peritoneal Exudate Cells and Spleen Lymphocyte Total Lipids of Rats[a]

Cells and fatty acid	Group		
	Control	0.5% CLA	1.0% CLA
	(wt%)		
Peritoneal exudate cells			
18:2n-6	5.5	5.3	4.2
20:3n-6	0.8	0.7	n.d.
20:4n-6	12.7	11.3	9.0
22:4n-6	5.6	5.3	4.2
22:6n-3	0.6	0.6	0.5
CLA			
9t,11c/9c,11t	n.d.	0.1	0.2
10t,12c	n.d.	0.2	0.2
Spleen lymphocytes			
18:2n-6	12.2 ± 0.8	10.4 ± 0.9	9.3 ± 0.9
20:3n-6	1.6 ± 0.2	1.3 ± 0.3	0.9 ± 0.1
20:4n-6	20.2 ± 0.8[a]	15.4 ± 1.3[a,b]	14.7 ± 1.7[b]
22:4n-6	2.5 ± 0.1	2.0 ± 0.2	1.9 ± 0.2
22:6n-3	1.2 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
CLA			
9t,11c/9c,11t	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
10t,12c	n.d.	0.2 ± 0.0	0.2 ± 0.0

[a]Values are means of two pooled samples from two and three rats each for the exudate cells, and means ± SE of three, five, and five rats for control, 0.5% CLA, and 1.0% CLA, respectively. Values without a common superscript letter (a,b) are significantly different at $P < 0.05$; n.d., not detected. For other abbreviation see Table 1.

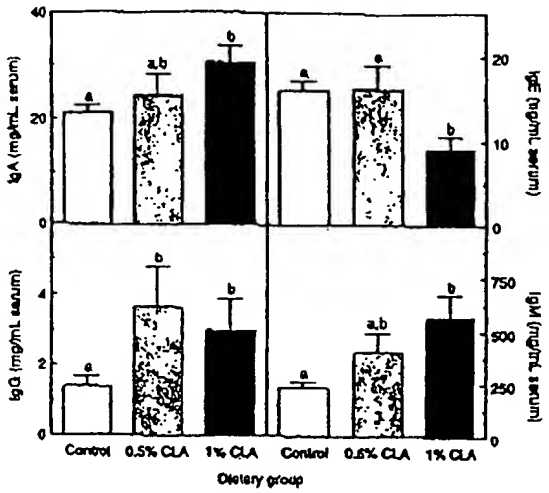

FIG. 5. Effect of dietary CLA on the concentration of serum immunoglobulins (Ig). Mean ± SE of five rats. Values without a common letter (a,b) are significantly different at $P < 0.05$. For abbreviation see Figure 1.

DISCUSSION

The pathway from linoleate to arachidonate and then eicosanoids is crucial to a range of metabolic diseases (28,29). Food allergy is one such disorder, and it is known that some eicosanoids are involved as chemical mediators in the manifestation of clinical symptoms of hypersensitivity (12,13). The inhibitors of LT production have now been clinically adopted (30,31). However, less is known of the effect that food components exert on this process. Although several food components have been shown to reduce eicosanoid production *in vitro*, in most cases it is practically unsatisfactory because of the limited efficacy (21,22). The results of the present study showed that CLA effectively controlled the production of LTB_4, LTC_4, and PGE_2. CLA significantly reduced LTC_4 production in the lung but not in the spleen. A similar tissue-specific reduction of LTC_4 was observed in rats given sesamin and α-tocopherol simultaneously, while in the spleen LTB_4 but not LTC_4 was reduced (21,22). These observations suggest a complex interaction between dietary fat and antioxidants in the LT-producing system.

Numbers of animal studies showed that dietary PUFA effectively modify the production of eicosanoids, and there is an interaction between n-6 and n-3 PUFA (32). PUFA of the n-3 family suppress the production of eicosanoids from arachidonic acid and exert a substantial suppressing effect on carcinogenesis in breast and colon (33,34). However, the anticarcinogenic effect of n-3 PUFA is far less than that of CLA (2–4). Eicosanoid production is known to be dependent on

TABLE 4
Effects of CLA on the Immunoglobulin Production in Splenic and Mesenteric Lymph Node Lymphocytes of Rats[a]

Immunoglobulin	Without lipopolysaccharide			With lipopolysaccharide		
	Control	0.5% CLA	1% CLA	Control	0.5% CLA	1% CLA
Spleen (ng/mL)						
IgA	3.75 ± 1.23	4.83 ± 0.99	3.78 ± 0.96	9.74 ± 2.45	13.6 ± 3.27	8.30 ± 2.50
IgG	51.0 ± 4.6	53.8 ± 2.3	61.5 ± 2.8	68.1 ± 2.4	71.9 ± 1.9	74.4 ± 1.9
IgM	223 ± 22	228 ± 6	246 ± 9	311 ± 9[A]	348 ± 8[B]	394 ± 6[C]
IgE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Mesenteric lymph node (ng/mL)						
IgA	1.65 ± 0.13[a]	4.78 ± 1.77[b]	5.05 ± 0.10[b]	2.91 ± 0.23[A]	8.72 ± 0.90[B]	22.3 ± 0.7[C]
IgG	n.d.	3.08 ± 0.69[a]	28.1 ± 4.38[b]	n.d.	4.64 ± 0.11[A]	31.9 ± 4.1[B]
IgM	1.86 ± 0.34[a]	4.74 ± 0.50[a]	96.6 ± 13.4[b]	2.85 ± 0.44[A]	6.36 ± 0.48[B]	122 ± 9[C]
IgE	3.81 ± 0.32	4.02 ± 0.33	3.64 ± 0.47	4.81 ± 0.17[A]	4.52 ± 0.29[A]	3.74 ± 0.21[B]

[a]Means ± SE of five rats. Values without a common superscript letter (A,B,C,a,b,c) are significantly different at $P < 0.05$. The lymphocytes were incubated with or without lipopolysaccharide for 72 h, and the concentration of immunoglobulins (Ig) in the supernatant was measured; n.d., not detected.

the substrate availability (35). CLA reduced the proportion of n-6 PUFA including arachidonic acid in the immune cells as observed in the liver and other tissues (8,14). Because of the limited availability of PEC samples for fatty acid analysis, they were analyzed as two pooled samples from two and three rats each. Though the number of analysis may not permit us to draw a definite conclusion, it seems likely that fatty acid composition of PEC also responded similarly as in spleen lymphocytes. This reduction was at least responsible for the reduced production of LT and PG in these cells. CLA may affect metabolic interconversion of fatty acids in the liver that may ultimately result in modified fatty acid composition and arachidonate-derived eicosanoid production in extrahepatic tissue (14). However, more direct participation of the metabolites of CLA cannot be ruled out (9,36). Therefore, the present study added possible usefulness of CLA for controlling the allergic reaction caused by food. Since the effect of CLA on Ig production differed between MLN lymphocytes and spleen lymphocytes, the analysis of the fatty acid composition of the former cells may provide a clue to understanding the mechanism of action.

In contrast to the eicosanoid production, the level of histamine released from PEC, which reflects the mast cell degranulation by a receptor-independent pathway, apparently was not modified by CLA and more directly the fatty acid composition of membrane phospholipids. Engels *et al.* (37) observed that the type of dietary fats and thus the change in the fatty acid composition of mast cell phospholipids did not influence the cell degranulation process. CLA is reported to be incorporated into triglyceride more preferably than phospholipids in tumor cells (7). Thus, CLA may not substantially influence the fatty acid composition of membrane phospholipids and hence, the structure and function of the membrane. In such a situation, the degranulation of the mast cells may not be modified largely.

An interesting observation is that CLA regulates the Ig production class specifically. Food allergy reaction is initiated by the production of allergen-specific IgE (12,13). IgA, in contrast, serves as an antiallergic factor by interfering with the intestinal absorption of allergen, and IgG also works as an antiallergic factor through the competition with binding of allergen to the receptor on the surface of the target cells such as mast cells and basophiles (12,13). CLA increased the production of IgA and IgG, while reducing that of IgE in lymphocytes, in particular MLN lymphocytes irrespective of the presence or absence of lipopolysaccharide, a cell activator. The response of splenic lymphocytes to CLA was less clear except for a slight but significant increase in IgM after lipopolysaccharide activation. However, the response pattern similar to MLN lymphocytes was observed in serum, indicating that CLA can modify the Ig levels preferably even on a whole-body basis. Bile acids (24) and unsaturated fatty acids (25) also regulate antibody production class specifically, but in a manner contrasted from that of CLA. These compounds may promote the allergic response through an increase in IgE production and a reduction in IgA and IgG production. It is plausible that the production of IgE and of IgA and IgG are at least reciprocally regulated. Thus, in addition to the favorable effect on the eicosanoid production, CLA was expected to mitigate the food allergic reaction.

The amounts of CLA ingested by rats of the present study corresponded to approximately 30 and 60 mg/100 g body weight for 0.5 and 1.0% CLA diets, respectively. These amounts were pharmacological when extrapolated to human, 18 and 36 g/60 kg body weight/day. However, as in the case of weight reduction in man, approximately 3 g/d for 2 to 3 mon, a prolonged ingestion may produce a favorable effect even at a lower dose. A long-term trial with a lower dietary level of CLA merits further study.

In conclusion, CLA produced a situation favorable for mitigation of food allergic reaction. Since the effect was seen at a dietary level as low as 0.5 or 1.0%, it is likely that CLA can strongly regulate multiple metabolic processes. Thus, the clinical application of CLA is warranted. Studies with immunized animals will provide more direct information regarding this issue.

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